REMARKS

Claims 97, 101-111 and 113-118 are pending in the application. These claims have all been cancelled without disclaimer or prejudice to applicants' right to pursue patent protection for the subject matter thereof in a subsequent application. New claims 119-143 are submitted herewith for the Examiner's review and consideration. These claims are completely supported by the application as filed (see below) and thus they raise no issue of new matter. Therefore, entry of this Amendment into the file of the application is respectfully requested such that claims 119-143 will be pending.

In particular, support for the new claims 119-143 may be found, *inter alia*, in the specification as follows: claim 119: page 5, lines 4-7, page 11, lines 13-24, page 32, lines 1-20, page 65, lines 9-15, page 76, lines 19-21 and Figure 1; claim 120: page 12, lines 15-16; claims 121-124: page 13, lines 8-26; claims 125-127: page 14, lines 1-5; claim 128: page 53, line 35 to page 54, line 4; claim 129: see support for claims 119-128; claim 130: pages 77-78 and the Experimental Results; claim 131: page 5, lines 4-7, page 11, lines 13-15, page 15, lines 11-22, page 32, lines 1-20, page 65, lines 9-15, page 76, lines 19-21 and Figure 1; claim 132: page 5, lines 4-7, page 11, lines 13-15, page 15, line 26 to page 16, line 20, page 32, lines 1-20, page 65, lines 9-15, page 76, lines 19-21 and Figure 1; claims 133-135: page 17, lines 5-10; claims 136-137: page 18, lines 5-10; claims 138-139: page 55, lines 10-14; claim 140: page 93, lines 1-3; claim 141: page 54, lines 19-21, page 66, lines 4-14 and page 87, lines 27-30; claim

142: page 54, lines 19-21 and claim 143: page 5, lines 4-7, page 11, lines 13-15, page 15, line 26 to page 16, line 20, page 32, lines 1-20, page 65, lines 9-15, page 76, lines 19-21, pages 77-78, Figure 1 and the Experimental Results.

Claim Rejections Withdrawn

Applicants note with appreciation the Examiner's statement in ¶4 on p. 2 of the Office Action that the rejection of claims 97, 101-111, and 113-118 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention, is withdrawn in view of the amendment to the claims.

The Examiner further stated in ¶5 on p. 2 of the Office Action that the rejection of claims 97, 101-111, and 113-118 under 35 U.S.C. 112, first paragraph as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention, is withdrawn in view of the arguments presented in the amendment.

Objection to the Disclosure

The Examiner stated in ¶6 on p. 3 of the Office Action that the prior objection to the disclosure is maintained for the reasons as set forth in the Office Action mailed June 19, 1998 (Paper No. 16). The Examiner further stated that applicants submit they will provide a new Figure 6B to overcome the rejection when the case is in condition for allowance. The

Examiner additionally stated that until applicants submit a proper Figure the objection is maintained.

In response to the above Objection, applicants submit that they will provide a new Figure 6B upon indication of allowable subject matter.

Obviousness-Type Double Patenting Rejections

The Examiner has provisionally rejected claims 97, 101-111 and 113-118 as unpatentable due to obviousness-type double patenting over claims 78-92 and 94-99 of copending Application No. 08/477,097 for the reasons made of record in Paper No. 20, mailed October 6, 1999, and Paper No. 22, mailed June 27, 2000. The Examiner states that applicants argue only that the rejection should be withdrawn if the claims are found to be allowable.

The Examiner has also provisionally rejected claims 97, 101-111 and 113-118 as unpatentable due to obviousness-type double patenting over claims 78-93 and 95-100 of application No. 08/475,084 for the reasons made of record in Paper No. 20, mailed October 6, 1999 and Paper No. 22, mailed June 27, 2000. The Examiner stated that applicants argue only that the rejection should be withdrawn if the claims are found to be allowable.

The Examiner has additionally provisionally rejected claims 97, 101-111 and 113-118 as unpatentable due to obviousness-type double patenting over claims 109-122 of copending Application No. 08/477,147. The Examiner stated that although

the claims are not identical, they are not patentably distinct from each other because the claims of 08/477,147 also encompass the same composition as that which is instantly claimed (a conjugate comprising a ganglioside derivative with an altered ceramide portion conjugated to an immunogenic protein based carrier, a saponin, and a pharmaceutically acceptable carrier, and a method of treatment using such). The Examiner stated that the rejection is maintained for the reasons of record, as applicant argues only that the rejection should be withdrawn if the claims are found allowable.

The provisional double-patenting rejections of claims 97, 101-111 and 113-118 over claims of Application Nos.08/477,097; 08/475,084 and 08/477,147 are respectfully traversed. In response to these rejections, applicants respectfully submit that M.P.E.P. §804 IB, in discussing provisional double-patenting rejections between copending applications, requires that the:

> 'provisional' double patenting rejection should continue to be made by the examiner in each application as long as there are conflicting claims in more than one application unless that 'provisional' double patenting rejection is the only rejection remaining in one of the applications. If the 'provisional' double patenting rejection in one application is the only rejection remaining in the application, the examiner should then withdraw that rejection and permit the application to issue as a patent, thereby converting the 'provisional' double patenting rejection in the other application into a double patenting rejection at the time one application issues as a patent. (emphasis supplied by applicants).

Applicants submit, therefore, that for the reasons discussed

below, new claims 119-143 are believed to distinguish the invention over all of the references cited by the Examiner to reject claims 97, 101-111 and 113-118, which rejections should therefore be withdrawn. Following such withdrawal, the only remaining rejection in this application would be the double-patenting rejection of the claims. In accordance with the M.P.E.P. section quoted above, the provisional double-patenting rejection should thus be withdrawn to permit the application to issue as a patent. Such action is therefore respectfully solicited.

New Grounds of Rejection

The Examiner stated in ¶10 at pages 5-7 of the Office Action that claims 97, and 101-110 are rejected under 35 U.S.C. 103(a) as being unpatentable over Wiegand et al U.S. Patent 5,599,914; issued Feb. 4, 1997; filed Nov. 24, 1989 ("Wiegand") in view of Fiume et al., Critical Rev. Therapeutic Drug Carrier Systems, 4(4):265-284, 1998 ("Fiume"), Ritter et al., Seminars in Cancer Biology, 2:401-409, 1991 ("Ritter"), Kensil et al., The Journal Of Immunology, 146(2):431-437, 1991 ("Kensil"), Marciani et al., Vaccine, 9:89-96, 1991 ("Marciani") and Uemura et al., J. Biochem, 79(6):1253-1261, 1976 ("Uemura").

The Examiner stated that Wiegand discloses modified glycosphingolipids (GM3, GD3, GM2 and GM1). The Examiner stated that Wiegand discloses a method for chemical modification of the sphingoid portions of glycosphingolipids to make glycosphingolipids capable of coupling to proteins, citing to the abstract of the reference. The Examiner stated

that Wiegand discloses that the method of chemical modification is that of ozonolysis at the C-4 double-bond of the sphingosine base resulting in the formation of a reactive aldehyde species (citing to col. 2, line 43-col. 3, line 67). The Examiner stated that Wiegand discloses that the aldehyde group is susceptible to reductive amination. The Examiner further stated, however, that Wiegand fails to disclose conjugation of the modified glycosphingolipid to KLH via an amine linkage between the C-4 carbon of sphingosine base and an ε-aminolysyl group of KLH. The Examiner additionally stated that Wiegand also fails to disclose a composition that comprises a saponin derivable from the bark of the Quillaja saponaria Molina tree (QS-21).

The Examiner stated that Fiume teaches that reductive amination of reactive aldehyde species with proteins having ε-lysine groups is well known in the art (citing to pages 268-269). The Examiner stated that specifically, Fiume teaches that the aldehyde group of a galactosyl residue may be reacted with a ε-lysine of a protein.

The Examiner stated that Ritter teaches that IgG responses to gangliosides may be increased by the covalent attachment of foreign carrier proteins such as KLH to the gangliosides, resulting in the T cell help necessary for the response (citing to page 406, paragraph 1). The Examiner stated that Ritter teaches that the advantage of inducing an IgG antibody response (versus IgM) against gangliosides is that IgG: a) has a higher affinity, b) is better able to penetrate solid tissues, c) is able to mediate antibody-dependent cell-

mediated cytotoxicity, and d) is generally detectable in the serum for longer periods after immunization.

The Examiner stated that Kensil et al teach that QS-21 (i.e., the instant carbohydrate derivable from the bark of a Quillaja saponaria Molina tree) produced a higher antibody response than conventional aluminum hydroxide (citing to page 433, column 2, paragraph 4, and Figure 3). The Examiner stated that Kensil et al also teach that the immune responses obtained with QS-21 reached a plateau at doses between 10-80 µg in mice (citing to page 433, column 1, paragraph 3).

The Examiner stated that Marciani et al teach that the use of QS-21 adjuvant was useful because it did not cause a toxic reaction in cats (citing to page 93, paragraph 1).

The Examiner stated that Uemura et al teach that the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity of the ganglioside derivative with antibodies.

The Examiner thus concluded that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to have used the modified GM2 glycosphingolipids of Wiegand to make GM2 glycoconjugates that are the same as those claimed. The Examiner stated that Wiegand teaches a modified glycosphingolipid that has a reactive aldehyde group (at the C-4 position of the sphingosine base) that may be used for coupling to proteins as taught by Fiume, because Fiume demonstrates that methods of

reductive amination to link proteins, via ε-lysine residues, to reactive aldehyde groups is known in the art. The Examiner stated that because Wiegand teaches a method of ozonolysis that results in the formation of a reactive aldehyde species, the bond that would be formed between the C-4 carbon of the sphingosine base and the KLH would be an amino linkage that would case the C-4 carbon to be present in a CH_2 group. The Examiner then stated that it would have been further *prima facie* obvious to one of ordinary skill in the art to have used KLH as the protein carrier because, as Ritter teaches, the attachment of gangliosides to carrier proteins such as KLH increases IgG responses to gangliosides. The Examiner also stated that it would have been *prima* facie to one of ordinary skill in the art to add QS-21, because, as taught by Kensil, it provides for a higher antibody response, and QS-21 provides the advantages that it is not toxic to animals (citing to the Marciani reference).

The Examiner stated that it also would have been *prima facie* obvious to optimize the doses of QS-21 in the composition, and that it would have been *prima facie* obvious to one of ordinary skill in the art to optimize the weight ratio of the components of the composition to provide an optimal response.

The Examiner then stated that one would have reasonably expected the conjugation procedure to work as substituted because conjugation through the ε-aminolysyl groups of carrier proteins for enhancing immunogenicity is routine in the art and, as Uemura (1976) teaches, ozonolysis and reduction of various sphingolipids does not affect the haptenic reactivity

with antibodies.

Applicants respectfully traverse this new ground of rejection. The invention recited in the new claims 119-143 submitted herewith is not suggested to one of ordinary skill in this field of art by the disclosure of the above-cited references, whether they are taken alone or in combination, for the reasons set forth below.

Turning first to Wiegand, the subject reference is directed to the chemical modification of the sphingoid portions of glycosphingolipids and the subsequent coupling of such modified glycosphingolipids to other molecules, such as proteins. The glycosphingolipids useful in the process disclosed by the reference, however, are disclosed in a generic fashion with no teaching or suggestion that any particular species within this genus would perform more effectively than any other, i.e., when linked to form an immunoconjugate composition such as is taught and claimed in the present application. This broad scope of disclosure is specifically demonstrated in, for example, Example B of the reference (commencing at column 5), which describes, *inter alia*, the preparation and subsequent coupling of reductively aminated ozonolysis products of the gangliosides GM3, GD3, GM2 and GM1. No particular ganglioside is described as being preferred or as performing more effectively than any of the other gangliosides falling within the scope of the reference. In particular, there is no teaching or suggestion that the modification and conjugation of, specifically, a derivative of the GM2 ganglioside (as specifically recited in applicants'

new independent claims) would produce a composition having properties superior in any fashion to those obtained with the use of any other(s) of the gangliosides falling within the scope of the genus of glycosphingolipids disclosed in the reference.

Several additional features which further distinguish the invention from the Wiegand reference are pointed out by the Examiner herself in the Office Action. In particular, the Examiner stated (as noted above) on page 5 of the Office Action that: (i) Wiegand fails to disclose conjugation of the modified glycosphingolipid to KLH, (ii) Wiegand fails to disclose conjugation via an amine linkage between the C-4 carbon of sphingosine base and an ε-aminolysyl group of KLH, and (iii) Wiegand also fails to disclose a composition that comprises a saponin derivable from the bark of a Quillaja saponaria Molina tree (i.e., QS-21).

The presently claimed invention is thus distinguishable and therefore patentable over Wiegand on several grounds, as discussed below. First, the Wiegand reference contains no teaching or disclosure which would suggest to one of ordinary skill in this art to use, in particular, the GM2 ganglioside derivative specifically recited in applicants' claims, in an immunoconjugate of the present invention. Second, there is no disclosure in the Wiegand reference which teaches or suggests the conjugation of a (modified) glycosphingolipid with Keyhole Limpet Hemocyanin ("KLH"), i.e., the specific immunogenic protein recited in claims 119-143 now pending in the application. That is, the only particular example of an

immunogenic protein disclosed in Wiegand is human serum albumin ("HSA") (see, e.g., col. 5, lines 25-30). Third, there is no disclosure in Wiegand which would suggest the formulation of, specifically, a GM2-KLH conjugate with QS-21. As noted above, the Office Action states (see p. 5) that Wiegand fails to disclose a composition that comprises a saponin derivable from the bark of a Quillaja saponaria Molina tree, i.e., QS-21. Fourth, it is not obvious from the disclosure of the reference that a vaccine comprised of a conjugate of a GM2 ganglioside derivative coupled to KLH, and a saponin derivable from the bark of a Quillaja saponaria Molina tree would provide a superior immunogenic effect in human cancer patients.

More particularly, with regard to the issue of whether it would be obvious to one of ordinary skill in this art to couple a GM2 ganglioside derivative to KLH, the Examiner as noted above stated in the Office Action that Wiegand does not teach or suggest such a construct. The Examiner thus has cited the Ritter reference in an effort to supply this missing element. There is, however, no teaching in either the Wiegand reference or the Ritter reference to suggest the combination of their disclosures. The references, moreover, teach away from such a combination in that while Wiegand does disclose the conjugation of a modified glycosphingolipid to a carrier, the particular carrier taught for use by the patentee is human serum albumin ("HSA"), see, e.g., Example B at col. 5, lines 24-28. In contrast, Ritter discloses the use of Keyhole Limpet Hemocyanin ("KLH") as the immunogenic carrier protein. There is thus no support for the Examiner's proposed combination of

Wiegand and Ritter.

Further to the above, there is also no disclosure contained in the remaining cited references to suggest to one of ordinary skill in this art the formation of a GM2-KLH conjugate in combination with QS-21. The Examiner acknowledged in the Office Action (see p. 5) that Wiegand fails to disclose a composition comprising a saponin derivable from the bark of a Quillaja saponaria Molina tree. Ritter also contains no such disclosure. The Examiner thus cited the Kensil and Marciani references for their disclosure (noted above) concerning QS-21. Neither of these references, however, discloses the conjugation of a modified ganglioside derivative with an immunogenic protein carrier such as KLH. Moreover, as demonstrated by the Experimental Results described in the present application, the inclusion of the QS-21 adjuvant in the claimed composition provides unexpectedly improved results over those obtained with the use of other known adjuvants. Applicants submit, therefore, that these improved results evidence that the inclusion of the QS-21 adjuvant in a composition comprising the GM2 ganglioside derivative-KLH conjugate would not be obvious to one of ordinary skill in this art.

In summary, therefore, as recognized by the Examiner in her Office Action, and as further established by the arguments provided herein, the combination of Wiegand with any or all of the Ritter, Kensil and Marciani references neither teaches nor suggests the formation of a GM2-KLH conjugate in combination with QS-21, utilizing the specific mode of linking the

conjugate together as recited in the claims. For this reason, the invention as presently claimed is believed to patentably distinguish over the cited references.

Moreover, the Experimental Results portion of applicants' specification conclusively demonstrates that the presently claimed composition incorporating GM2 ganglioside derivatives is very successful in stimulating or enhancing the production of antibodies in human subjects so as to treat and/or prevent cancers, particularly melanoma, in such human subjects. Such a result could not have been predicted from the cited art. Applicants attach hereto as **Exhibit A** a copy of a paper by Ragupathi, et al., "Introduction of Antibodies Against GD3 Ganglioside in Melanoma Patients By Vaccination With GD3-Lactone-KLH Conjugate Plus Immunological Adjuvant QS-21", Int. J. Cancer: 85 659-666 (2000). Exhibit A clearly demonstrates that compositions based on the GD3 ganglioside-KLH +QS-21 are **NOT** effective in stimulating the production of GD3 antibodies in humans. In particular, the authors stated at p. 665, second column, ¶2, lines 18-19 that, "GD3-KLH failed to induce antibody against GD3". (emphasis supplied by applicants). Applicants submit, therefore, that the improved results obtained with the GM2 ganglioside derivatives conjugated to KLH (as recited in the claims) + QS-21, in contrast to the poor results achieved with a comparable GD3-KLH conjugate + QS-21, as described by the authors of the subject Exhibit, provide convincing evidence that the presently claimed invention would **NOT** be obvious to one of ordinary skill in this art over the cited references, whether such references are taken alone or in any combination.

Applicants therefore submit that for the preceding reasons the invention as presently claimed is distinguishable over the Wiegand, Ritter, Kensil and Marciani references cited in combination to reject claims 97 and 101-110. Whether the references are taken individually or in combination, the arguments above clearly support applicants' position that the invention as presently claimed is not obvious over the disclosure of the cited prior art. [1,2] Moreover, as discussed in the footnotes below, neither the Fiume nor the Uemura references, cited by the Examiner in combination with the Wiegand, Ritter, Kensil and Marciani references to reject claims 97 and 101-110, supply the elements missing from the above-discussed references in a manner so as to render the invention as now claimed obvious to one of ordinary skill in this art. The Examiner is thus respectfully requested to reconsider and withdraw her rejection of claims 97 and 101-110

---

1 The Fiume reference cited by the Examiner in combination with the other references discussed above to reject the claims relates to the placement of an aldehyde derivative on the sugar portion of a sphingolipid and linking this structure to a ε-amino group of lysine located upon a protein. The Examiner's suggestion on p. 6 of the Office Action that Fiume has a more generic teaching, i.e., that it teaches that reductive amination of reactive proteins having ε-lysine groups is well known in the art is not supported by the disclosure of the reference, which is limited to the chemistry of sugar-based compositions. Moreover, as shown, for example, in the paper by Ritter et al. attached hereto as **Exhibit B**, entitled, "Antibody Response To Immunization With Purified GD3 Ganglioside and GD3 Derivatives (Lactones, Amide and Gangliosidol) in the Mouse", Immunobiol.:182 32-43 (1990) it was well known in the art that at least as of the date of the reference the conjugation of ganglioside derivatives through the sugar portion did not provide a composition effective for stimulating or enhancing the production of antibodies (see, e.g., Table I on p. 34).

2 Further according to the Examiner (at p. 6 of the Office Action) the Uemura reference additionally cited in combination with the other above-discussed references to reject the claims teaches that the ozonolysis and reduction of various sphingolipids did not affect the haptenic activity of the ganglioside derivative with antibodies. The Ragupathi reference attached as **Exhibit A**, however, demonstrates that the Examiner's statement is not generally correct, since the GD3-based conjugate, formed with ozonolysis and reduction of the sphingolipid, "[f]ailed to induce antibody against GD3" (see p. 665), thus demonstrating that haptenic activity of certain sphingolipids can be negatively affected by such ozonolysis and reduction.

based on the references discussed above.

In ¶11 of the Office Action, claims 97, 101-111, 113 and 115-118 are rejected under 35 U.S.C. 103(a) as being unpatentable over Wiegand, Fiume and Livingston et al., Cancer Research, 149:7045-7050, 1989 ("Livingston") in view of Ritter, Livingston et al. U.S. Patent No. 5,102,663 ("Livingston '663 patent"), Kensil, Marciani and Uemura.

With regard to this rejection the Examiner stated that, as discussed above, Wiegand in combination with Fiume teaches a glycoconjugate as claimed in claim 97.

The Examiner additionally stated that Livingston teaches that melanoma recurrence was delayed in patients developing GM2 antibodies after treatment with the composition (citing to page 7048, paragraph 1 and column 2, paragraph 2). The Examiner stated that Livingston et al teach that more patients produced IgM antibodies than IgG antibodies to the GM2 (citing to page 7047, paragraph bridging columns 1-2). The Examiner additionally stated that Livingston et al. also teach the gangliosides GM2, GD2 and GD3 are expressed on the cell surface of human malignant melanomas (citing to page 7045, column 1, paragraph 2).

The Examiner stated that Ritter (1991) teaches that IgG responses to gangliosides may be increased by the covalent attachment of foreign carrier proteins such as KLH to the gangliosides, resulting in the T cell help necessary for the response (citing to page 406, paragraph 1). The Examiner

stated that Ritter teaches that the advantage of inducing an IgG antibody response (versus IgM) against gangliosides is that IgG: a) has a higher affinity, b) is better able to penetrate solid tissues, c) is able to mediate antibody-dependent cell-mediated cytotoxicity, and d) is generally detectable in the serum for longer periods after immunization.

The Examiner stated that the Livingston '663 patent teaches that gangliosides GM3, GM2, GD3, GD2, GT3 and O-acetyl GD3 are gangliosides that are prominent cell-membrane components of melanoma and other tumors of neuroectodermal origin (citing to column 1, lines 22-28).

The Examiner stated that Kensil et al teach that QS-21 (i.e., the instant carbohydrate derivable from the bark of a Quillaja saponaria Molina tree) produced a higher antibody response than conventional aluminum hydroxide (citing to page 433, column 2, paragraph 4, and Figure 3). The Examiner also stated that Kensil et al also teach that the immune responses obtained with QS-21 reached a plateau at doses between 10-80 µg in mice (citing to page 433, column 1, paragraph 3).

The Examiner further stated that Marciani et al teach the use of QS-21 adjuvant was because it did not cause a toxic reaction in cats (citing to page 93, paragraph 1).

The Examiner next stated that Uemura et al teach that the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity of the ganglioside derivative with antibodies.

The Examiner then stated that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to have used the modified GM2 glycosphingolipids of Wiegand to make GM2 glycoconjugates that are the same as those claimed, and then to have used the glycoconjugates in compositions for stimulating or enhancing antibody production or in a method of treating cancer, because Livingston teaches that melanoma recurrence is delayed in patients developing GM2 antibodies after treatment with vaccines comprising GM2 (citing to page 7048, paragraph 1 and column 2, paragraph 2). The Examiner stated that Livingston et al teach that more patients produced IgM antibodies than IgG antibodies to the GM2 (citing to page 7047, paragraph bridging columns 1-2). The Examiner stated that Livingston et al also teach the ganglioside GM2 is expressed on the cell surface of human malignant melanomas (citing to page 7045, column 1, paragraph 2). The Examiner stated that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to have added QS-21 as an adjuvant to the GM2-KLH conjugate for use as a vaccine because the conjugated composition would be expected to enhance the IgG response to the gangliosides, as taught by Ritter et al (1991), thus providing the advantages of Ritter et al (1991) and that, as Kensil teaches, adding the QS-21 is advantageous because it provides for a higher antibody response than the commonly used adjuvant. The Examiner additionally stated that, in addition, QS-21 provides the advantage that it is not toxic to animals (citing to the Marciani reference).

The Examiner then went on to state that it also would have been *prima facie* obvious to one of ordinary skill in the art to optimize the doses of QS-21 in the composition and that it would have been additionally *prima facie* obvious to one of ordinary skill in the art to optimize the weight ratio of the components of the composition to provide an optimal response.

The Examiner next stated that one would have reasonably expected the conjugation procedure to work as substituted because conjugation through the ε-aminolysyl groups of carrier proteins for enhancing immunogenicity is routine in the art and, as Uemura (1976) teaches, ozonolysis and reduction of various sphingolipids does not affect the haptenic reactivity with antibodies.

The Examiner stated that optimization of the dosage, route of immunization and number of sites of immunization to administer the composition is well within the skill of the ordinary artisan.

The Examiner then repeated that one would have reasonably expected the conjugation procedure to work as substituted because conjugation through ε-aminolysyl groups of carrier proteins for enhanced immunogenicity is routine in the art and Uemura et al (J. Biochem, 79(6):1253-1261, 1976) teach that the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity with antibodies.

Applicants respectfully traverse the above-cited ground for rejection of claims 97, 101-111, 113 and 115-118. In the

discussion above with respect to the previous ground of rejection, applicants established that their invention is patentably distinguishable over six of the eight references cited in combination to reject claims 97, 101-111, 113 and 115-118, i.e., Wiegand, Fiume, Ritter, Kensil, Marciani and Uemura. The arguments concerning those references, which are specifically incorporated herein by reference, are not repeated here. The two new references in the combination are Livingston et al., Cancer Research, 149: 7045-7050 (1989) ("Livingston") and Livingston et al. U.S. Patent No. 5,102,663 ("the Livingston '663 patent"). As discussed below, neither Livingston, nor the Livingston '663 patent, taken in combination with the other six references discussed above, would have rendered the invention as presently claimed obvious to one of ordinary skill in this field of art.

The Livingston reference was cited by the Examiner for its disclosure (1) concerning the effect of GM2 antibodies on melanoma recurrence, (2) that more patients produced IgM antibodies than IgG antibodies to the GM2, and (3) that the gangliosides GM2, GD2 and GD3 are expressed on the cell surface of human malignant melanomas. The reference does not, however, provide the elements missing from the disclosure of the six references discussed above, i.e., there is no teaching or suggestion in Livingston to form a conjugate comprising a conjugate of a GM2 ganglioside derivative to Keyhole Limpet Hemocyanin, in combination with a saponin derivable from the bark of a Quillaja saponaria Molina tree, wherein in the conjugate the ganglioside derivative is covalently bound to the Keyhole Limpet Hemocyanin by a stable amine bond between

the C-4 carbon of the altered sphingosine base of the altered ceramide portion of the ganglioside derivative and the nitrogen of the ε-aminolysyl group of Keyhole Limpet Hemocyanin. These features of the invention are found in each of the new independent claims of the application, including method claim 131 directed to a method of stimulating or enhancing production of an antibody using a GM2 ganglioside derivative, claim 132 directed to a method of treating a cancer in a subject and claim 143 directed to a method of delaying recurrence of melanoma in subjects at risk of relapse of melanoma, but are not found within the cited reference. Thus, as the Livingston reference fails to provide the elements of the invention missing from the cited prior art, new claims 119-143 are believed to distinguish the invention over the reference, whether taken alone or in the combination relied upon by the Examiner.

The Livingston '663 patent teaches that certain gangliosides (i.e., GM3, GM2, GD3, GD2, GT3 and O-acetyl GD3) are prominent cell-membrane components of melanoma and other tumors of neuroectodermal origin. In like manner to the Livingston reference discussed above, the Livingston '663 patent neither discloses nor suggests the compositions set forth in applicants' new claims, wherein the conjugate is linked in the manner recited, nor the methods claimed for using the claimed composition for stimulating or enhancing antibody production, for treating cancer and for preventing the relapse of melanoma in subjects at risk of such relapse. Therefore, the invention as recited in applicants' new claims 119-143 is clearly patentably distinct over the Livingston '663 patent taken

alone or in combination with the other cited references relied upon by the Examiner. The Examiner is thus respectfully requested to reconsider and withdraw the rejection of claims 97, 101-111, 113 and 115-118 based upon the cited combination of references.

In ¶12 of the Office Action, claim 114 is rejected under 35 U.S.C. 103(a) as being unpatentable over Wiegand in view of Fiume, Livingston, Ritter, the Livingston '663 patent, Kensil, Marciani and Uemura as applied to claims 78, 80-92, 94 and 96-99 above [sic., claims 97, 101-111, 113 and 115-118] and further in view of Irie et al. U.S. Patent No. 4,557,931 ("the Irie '931 patent").

With regard to this rejection the Examiner stated that the teachings of Wiegand, Fiume, Livingston et al (1989), Ritter et al. (1991), Livingston et al. (U.S. Patent No 5,102,663), Kensil (1991), Marciani (1991) and Uemura (1976) are already discussed in the Office Action. The Examiner stated that the combination differs [i.e., from the invention] by not teaching the administration of the composition for treating cancer of epithelial origin.

The Examiner stated that Irie et al teaches that the ganglioside GM2 is found on or in tumors of a variety of histological types including melanoma and breast carcinomas (citing to column 1, lines 28-31).

The Examiner stated that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the

invention was made to administer the GM-2-KLH conjugate/QS-21 composition or other ganglioside conjugate/QS-21 composition as combined *supra* to patients afflicted with or susceptible to a recurrence of cancer of an epithelial origin (i.e. breast carcinomas) because the ganglioside GM-2 is found in the stroma of the tumor as taught by Irie et al and one of ordinary skill in the art would expect that the antibodies produced by the composition react with the tumor and treat the disease.

Applicants respectfully traverse this rejection for the reasons provided below. The newly cited reference relied upon in the rejection of claim 114 is the Irie '931 patent. Each of the other references cited in combination with the Irie '931 patent to reject applicants' claim 114 is discussed and distinguished above. Thus, these arguments are not repeated here, although they are specifically incorporated into this discussion by reference thereto.

The Irie '931 patent is cited, as noted above, for its disclosure that the ganglioside GM2 is found on or in tumors of a variety of histological types, including melanomas and breast carcinomas. The cited reference, however, does not provide the elements missing from the references discussed above, i.e., it does not disclose or suggest the claimed composition comprising a conjugate covalently bound as recited in the claims, and also including a saponin derivable from the bark of a Quillaja saponaria Molina tree, or the method of using such composition to enhance or stimulate antibody production, to treat cancer, or to prevent relapse of melanoma

in patients at risk of such relapse. For this reason, the present claims (nos. 119-143) are believed to patentably distinguish over the Irie '931 patent, taken alone or in the combination relied upon by the Examiner to reject claims 97, 101-111, 113 and 115-118. The Examiner is thus respectfully requested to reconsider and withdraw the rejection of claim 114 under 35 U.S.C. §103 (a).

Summary

For the reasons set forth hereinabove, applicants respectfully request that the Examiner reconsider and withdraw the various grounds of objection and rejection set forth in the Office Action and earnestly solicit allowance of the now pending claims, i.e., new claims 119-143.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' attorneys invite the Examiner to telephone either of them at the number provided below.

No fee is believed to be due with this Amendment. However, should any fee be due, authorization is hereby given to charge the required amount of such fee to Deposit Account No. 03-3125

Respectfully submitted,

Mark A. Farley

John P. White
Registration No. 28,678
Mark A. Farley
Registration No. 33,170
Attorneys for Applicant(s)
Cooper & Dunham, LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Mark A. Farley 3/31/03

John P. White Date
Reg. No. 28,678
Mark A. Farley
Reg. No. 33,170

Int. J. Cancer: 85, 659–666 (2000)
2000 Wiley-Liss, Inc.

Publication of the International Union Against Cancer

INDUCTION OF ANTIBODIES AGAINST GD3 GANGLIOSIDE IN MELANOMA PATIENTS BY VACCINATION WITH GD3-LACTONE-KLH CONJUGATE PLUS IMMUNOLOGICAL ADJUVANT QS-21

Govindaswami RAGUPATHI[1,2]*, Michael MEYERS[2], Sucharita ADLURI[1], Lisa HOWARD[1], Cristina MUSSELLI[1] and Philip O. LIVINGSTON[1,2]

[1]*Laboratory of Tumor Vaccinology, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*
[2]*Clinic Immunology Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*

The gangliosides GD3, GD2 and GM2 are expressed on the cell surface of malignant melanomas, GD3 being the most abundant. We have shown that immunization of melanoma patients with GM2 adherent to Bacillus Calmette-Guerin (GM2/BCG) induced an IgM antibody response. Vaccines containing GM2-keyhole limpet hemocyanin (KLH) conjugate and the immunological adjuvant QS-21 induced a higher titer IgM response and consistent IgG antibodies. Patients with antibodies against GM2 survived longer than patients without antibody. On the other hand, our previous trials with GD3/BCG, GD3 derivatives including GD3-lactone (GD3-L)/BCG failed to induce antibodies against GD3. In our continuing efforts to induce antibody against GD3, we have immunized groups of 6 melanoma patients with GD3-KLH or GD3-L-KLH conjugates containing 30 μg of ganglioside plus 100 μg of QS-21 at 0, 1, 2, 3, 7 and 19 weeks. Prior to vaccination, no serological reactivity against GD3 or GD3-L was detected. After immunization, IgM and IgG antibodies were detected against both GD3 and GD3-L in the GD3-L group exclusively. The GD3-L-KLH vaccine induced IgM titers against GD3-L of 1:40–1/1,280 in all patients and IgG titers of 1/160–1/1,280 in 4 patients. These antibodies also strongly cross-reacted with GD3. ELISA reactivity was confirmed by immune thin-layer chromatography on GD3 and melanoma extracts. Sera obtained from 4 of these 6 patients showed cell surface reactivity by FACS and from 2 showed strong cell surface reactivity by immune adherence (IA) assay and complement lysis against the GD3 positive cell line SK-Mel-28. *Int. J. Cancer* 85: 659–666, 2000.
2000 Wiley-Liss, Inc.

Gangliosides are sialic acid containing glycosphingolipids composed of a carbohydrate moiety attached to ceramide. Gangliosides GM2, GD2 and GD3 are expressed on the cell surface of human malignant melanomas and other tumors of neuroectodermal origin. These antigens have been demonstrated to be susceptible targets for treatment with monoclonal antibodies (MAbs) and vaccines (Jurcic *et al.*, 1997; Livingston, 1995; Ragupathi, 1996; Scheinberg and Chapman, 1995). We and others have shown that the presence of antibodies against GM2 (either naturally or vaccine induced) has been associated with an unexpectedly favorable disease-free and overall survival. We demonstrated that the optimal way to immunize against GM2 is by vaccinating with GM2 covalently conjugated to keyhole limpet hemocyanin (GM2-KLH) plus immunological adjuvant QS-21 (Helling *et al.*, 1995). However, GD3 is the dominant melanoma ganglioside and we have been unable to induce antibodies against GD3 in melanoma patients by active immunization with GD3-expressing melanoma cells or purified GD3 or GD3 congeners such as GD3-lactone plus Bacillus Calmette-Guerin (GD3-L/BCG) (Ritter *et al.*, 1991). However, GM3-L has been reported to be a more effective immunogen than GM3 (Nores *et al.*, 1987), presumably due to the increased molecular rigidity resulting from lactone ring formation, and the KLH conjugate vaccine is more immunogenic than the previous BCG vaccine. Consequently, we have tested the immunogenicity in melanoma patients of GD3-KLH plus QS-21 and GD3-L-KLH plus QS-21 vaccines and report the successful induction of antibodies against purified GD3 and melanoma cells expressing GD3 in the majority of patients.

MATERIAL AND METHODS

Material

GD3 extracted from bovine buttermilk was received from Matreya (Pleasant Gap, PA). GM1, GM2, GM3 and GD2 extracted from bovine brain, BSA, sodium cyanoborohydride, 4-chloro-1-naphtol and *p*-nitrophenyl phosphate were purchased from Sigma (St. Louis, MO). QS-21 (Kensil *et al.*, 1991) was obtained from Aquila (Framingham, MA). Clinical grade KLH was obtained from Perlmmune (Rockville, MD). Goat anti-human IgG and IgM conjugated with alkaline phosphatase obtained from Kierkegaard and Perry (Gaithersburg, MD) were used for ELISA. Goat anti-human IgM or IgG labeled with fluorescein isothiocyanate (FITC) were obtained from Southern Biotechnology Associates (Birmingham, AL) and used in a fluorescence-activated cell sorter (FACS). Horseradish peroxidase-conjugated goat anti-human IgM and IgG purchased from TAGO (Burlingame, CA) was used for dot-blot immune staining and immune thin-layer chromatography (ITLC). Rabbit anti-mouse immunoglobulins conjugated with horseradish peroxidase were obtained from Zymed (San Francisco, CA) and used for ITLC with mouse control MAb R24 against GD3 (Pukel *et al.*, 1982). High performance thin-layer chromatography (HPTLC) silica gel plates were obtained from Merck (Darmstadt, Germany).

Vaccine preparation

GD3-KLH conjugate was prepared as described previously (Fig. 1a) (Helling *et al.*, 1994). The principle involved in the conjugation procedure is cleavage of the double bond of ceramide by ozone, generation of an aldehyde group and conjugation to ε-amino groups on lysine of KLH by reductive amination. The GD3-KLH conjugate was prepared in 3 batches. The amount used for conjugation, the percent recovery and the GD3/KLH epitope ratio for the GD3-L-KLH vaccine are summarized in Table I. More than 23% of GD3 in the reaction mixture was conjugated with KLH. The GD3/KLH epitope ratio for the combined preparation was 1,049.

GD3-L-KLH vaccine. Because of the unstable nature of GD3-L, we firstly prepared the GD3-KLH conjugate then converted it to GD3-L-KLH by acid treatment (Fig. 1b) and lyophilized immediately. Briefly, equal volumes of GD3-KLH and glacial acetic acid (v/v) were mixed in a sterile glass tube. To monitor GD3-L conversion, the conversion of free GD3, a small portion of which had not been removed completely from the conjugation reaction, was determined by TLC. After 4 hr at 37°C with gentle shaking, when about

Grant sponsor: NIH; Grant number: CA 33049; Grant sponsor: the Koodish vaccine fund.

Contact address for M. Meyers: Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA.

*Correspondence to: Department of Medicine, Box 113, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA. Fax: (212) 794-4352. E-mail: raguparg@mskcc.org

Received 30 August 1999; Revised 23 September 1999

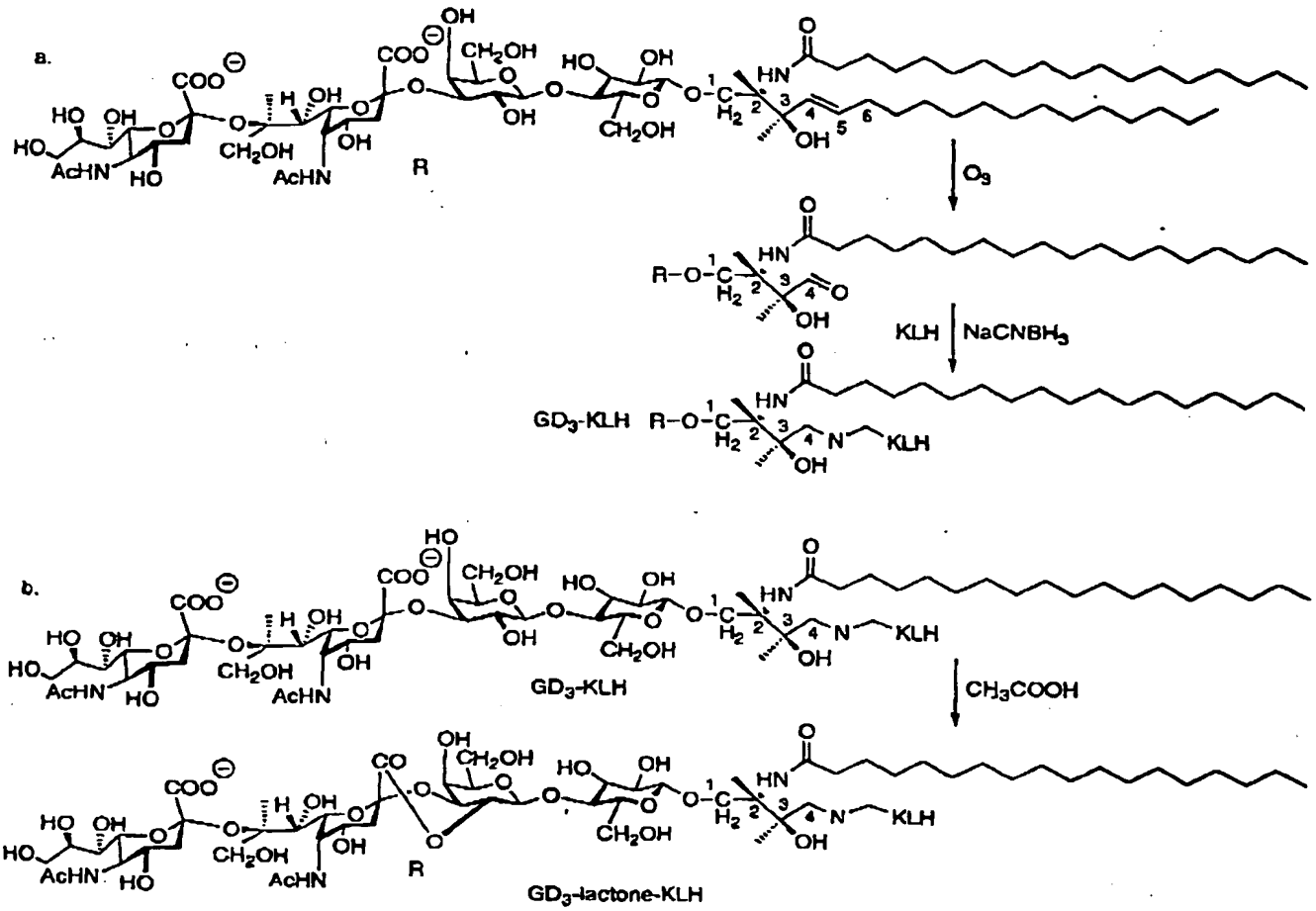


FIGURE 1 – (*a*) Synthesis of GD3-KLH conjugate after ozone cleavage and reductive amination. (*b*) Conversion of GD3-KLH conjugate to GD3-L-KLH conjugate.

TABLE 1 – PREPARATION AND ANALYSIS OF THE GD3-KLH CONJUGATE USED TO PREPARE THE GD3-KLH AND GD3-L-KLH VACCINES

Batch number[1]	Amount used for conjugation (mg)		Ratio by weight GD3:KLH	The amount of GD3 and KLH in conjugate (mg)		Recovered (%)		Epitope ratio of conjugate GD3/KLH
	GD3	KLH		GD3	KLH	GD3	KLH	
1	5.0	7.5	1:1.5	0.90	5.04	18.0	67.2	1,076.0
2	3.0	5.0	1:1.6	0.78	4.08	26.0	81.6	1,118.2
3	5.0	6.0	1:1.2	0.99	6.00	19.8	100	952.5
Combined	13.0	18.5	1:1.4	2.67	15.12	20.5	81.7	1,048.9

[1]All 3 batches were combined to prepare GD3-KLH and GD3-L-KLH conjugate vaccine (see Material and Methods).

80% of the GD3 had been lactonized, the acetic acid was quickly removed using a Centriprep (Amicon, Beverly, MA; 30 kDa molecular cut-off filter) with multiple saline washes. The conjugate was sterilized by passing through a 0.22-μm filter. The amount of GD3 or GD3-L in the conjugate was determined by estimating the sialic acid content by the resorcinol method (Svennerholm, 1963). GD3-KLH or GD3-L-KLH conjugate containing 30 μg ganglioside was aliquoted to individual vials and lyophilized under sterile conditions. In both cases, prior to the injection, 100 μg of QS-21 were mixed with the vaccine as it was reconstituted in normal saline solution.

Patients and clinical protocol

Patients with AJCC stage III or IV metastatic malignant melanoma (regional or systematic metastases) who were free of detectable melanoma within 2 weeks to 6 months after surgery were candidates for this trial. No patient had received prior chemotherapy. GD3-KLH or GD3-L-KLH conjugate containing 30 μg of ganglioside and 100 μg immunological adjuvant QS-21 were mixed immediately prior to vaccine administration in a total volume of 1 ml saline. Four vaccinations were administered s.c. at 1-week intervals, 2 additional vaccinations were administered at

TABLE II - PEAK ANTIBODY RESPONSE OF PATIENTS AFTER VACCINATION WITH GD3-KLH OR WITH GD3-L-KLH AS DETERMINED BY ELISA

Patient	Peak reciprocal ELISA titer							
	GD3				GD3-L			
	IgM		IgG		IgM		IgG	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
GD3-KLH lyophilized								
1	0	0	0	0	NT[1]	NT	NT	NT
2	0	0	0	0	NT	NT	NT	NT
3	20	80	0	80	NT	NT	NT	NT
4	0	0	0	0	NT	NT	NT	NT
5	0	0	0	0	NT	NT	NT	NT
6	0	20	0	20	NT	NT	NT	NT
GD3-L-KLH lyophilized								
1	0	40	0	160	0	40	0	160
2	0	0	0	0	0	40	0	0
3	0	40	0	20	0	160	0	0
4	0	160	10	1,280	0	640	0	1,280
5	0	40	0	160	0	40	0	160
6	0	1,280	0	320	0	1,280	0	1,280

[1]Not tested, because of the absence of antibodies against GD3 antibody.

intervals of 7 and 19 weeks from the date of first vaccination, under an MSKCC IRB approved protocol. Peripheral blood (20–30 ml) was drawn immediately before each vaccination, and 2 weeks after the 4th, 5th and 6th vaccinations.

ELISA

ELISAs were performed as described previously (Helling *et al.*, 1995). To determine the titers of GD3 and GD3-L antibodies, ELISA plates were coated with GD3-L or GD3 at 0.1 μg per well in ethanol. Serially diluted patient serum was added to wells of the coated plate and incubated for 1 hr at room temperature. Rabbit anti-human IgM or IgG conjugated with alkaline phosphatase served as second antibodies. The antibody titer was defined as the highest serum dilution showing an absorbance 0.1 or greater over that of normal sera. Immune sera were also tested for non-specific "stickiness" on plates that were processed identically but without ganglioside, and reading was subtracted from the value obtained in the presence of gangliosides.

Immune thin layer chromatography (ITLC)

Immune staining of gangliosides with MAbs or human sera was performed after separation of purified gangliosides or melanoma extracts on HPTLC silica gel glass plates as described previously (Hamilton *et al.*, 1993). The plates were coated with 1% Plexigum (Polyscience, Warrington, PA) in n-hexane, blocked with 3% BSA in PBS for 2 hr and incubated with patient sera (diluted 1:150 in PBS) overnight at room temperature. The plates were washed with PBS containing 0.05% Tween 20 (Fisher Scientific, Fair Lawn, NJ) and incubated with anti-human IgG or IgM antibodies conjugated with horseradish peroxidase at 1:200 dilution for 3 hr at room temperature. The plates were then washed with PBS-0.05% Tween 20 and developed with 4-chloro-1-naphtol-H_2O_2.

Dot-blot immune stain

Gangliosides GD3, GD3-L, GD2, GM1, GM2, GM3 and fucosyl (Fuc)-GM1 (0.1 μg) were spotted on nitrocellulose strips. The unreacted sites were blocked with 3% HSA-0.05% Tween-20 in PBS. The strips were treated as described for ITLC except coating with Plexigum. The intensity of spots in dot-blot immune stains was graded -, +, ++ or +++.

Fluorescence activated cell sorter (FACS) assay

The GD3 positive melanoma cell line SK-MEL-28 served as a target. Single cell suspensions of 2×10^5 cells per tube were washed with 3% FCS in PBS and incubated with 20 μl of 1:20 diluted antisera or MAb R24 for 30 min on ice. After washing the cells twice with 3% FCS in PBS, 20 μl of 1:15 rabbit anti-human IgG or IgM-labeled with FITC were added. The suspension was mixed, incubated for 30 min and washed. The percent positive population and mean fluorescence intensity of stained cells were analyzed using a FACS Scan (Becton-Dickinson, Mountain View, CA) (Zhang *et al.*, 1995).

Immune adherence (IA) assay

The IA assay measures rosetting of human RBC (blood group O) with guinea pig complement on target cells (SK-MEL-28) mediated by complement binding antibodies, and was performed as described previously (Shiku *et al.*, 1976). Individual target cells were scored as positive when 50% or more of the cell perimeter was surrounded by indicator cells.

Complement-dependent cytotoxicity (CDC)

CDC was assayed at a serum dilution of 1:10 with SK-MEL-28 cells and human complement by a chromium-release assay as previously described (Helling *et al.*, 1995). All assays were carried out in triplicate. Cells incubated only with culture medium, complement, antisera or MAb R24 served as controls. Spontaneous release was calculated based on the chromium released by target cells incubated with complement alone. Maximum release was determined by incubating target cells with complement and 1% Triton X-100. Percent cytolysis was calculated according to the formula:

$$\text{Specific release (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Inhibition assay

Antisera at 1:150 dilution or MAb R24 at 1 μg/ml were mixed with various concentrations of structurally related and unrelated ganglioside antigens. The mixture was incubated at room temperature for 30 min and tested on a GD3-coated plate by ELISA. Percentage inhibition was calculated as the difference in absorbance (ELISA) between the uninhibited and inhibited serum.

RESULTS

Clinical considerations

All patients signed informed consent prior to vaccination. Complete blood counts (CBC), liver function tests and clinical evaluations were repeated at 2-month intervals. Toxicity was restricted

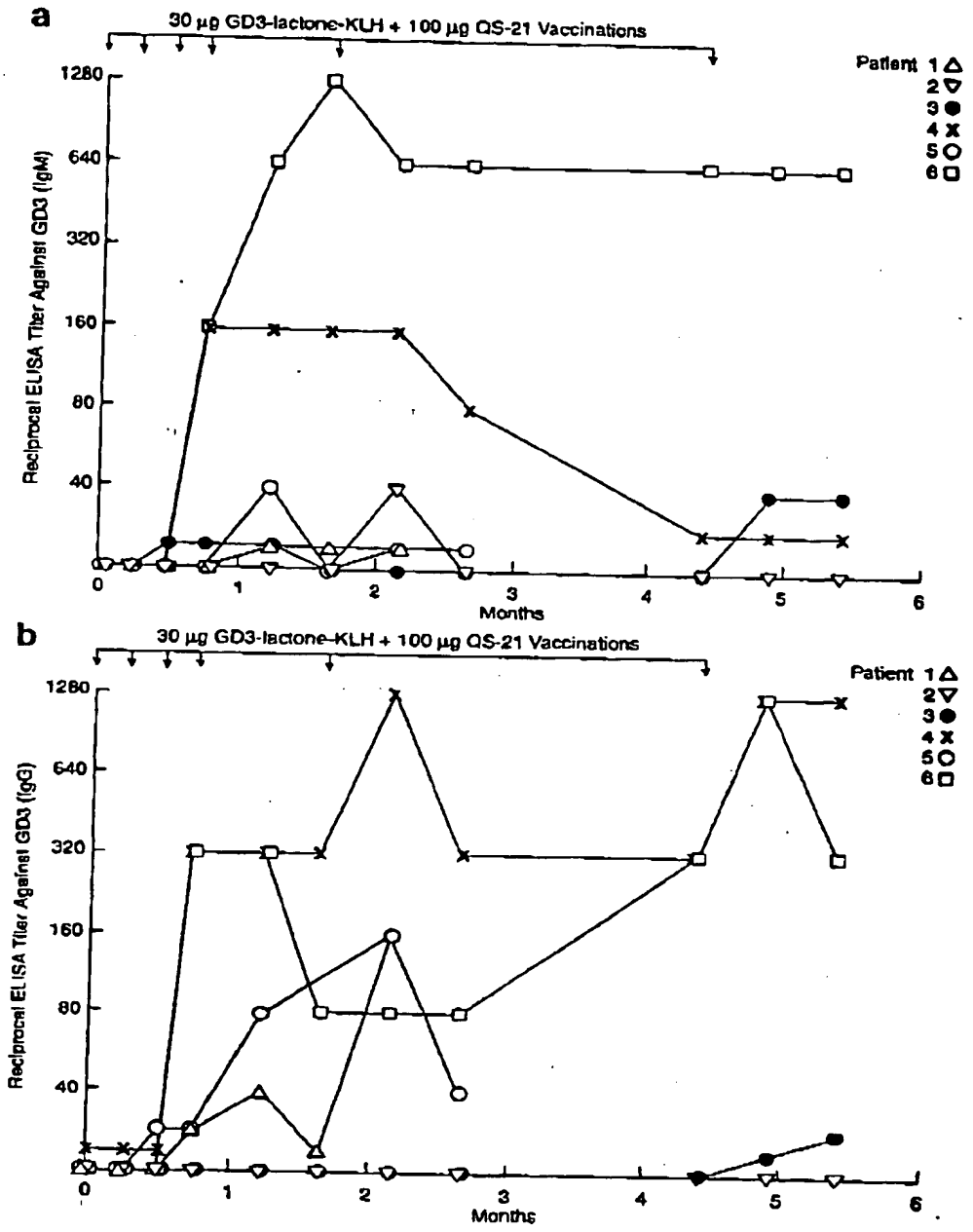


FIGURE 2 – Time course of the induction of IgM *(a)* and IgG *(b)* antibodies in 6 patients immunized with GD3-L-KLH plus QS-21.

to grade II local erythema and induration at vaccination sites lasting 3–5 days in all patients and grade I fever and flu-like symptom lasting 1–2 days after 2–3 immunizations in one half of the patients. This is the pattern of side effects associated with QS-21 administration at the 100-μg dose (Livingston *et al.*, 1994). No other side effects were detected.

ELISA responses against GD3 and GD3-L

The ELISA titers against GD3 and GD3-L are summarized in Table II. Before vaccination, IgG antibodies against GD3 or GD3-L were detected in 1 patient (titer 1/10) and IgM antibodies were detected at a titer of 1/20 in 1 patient. After vaccination, only one patient vaccinated with GD3-KLH developed detectable IgM and IgG antibodies against GD3 (titers 1/80). Consequently, further analysis was not carried out with GD3-KLH sera. The GD3-L-KLH vaccines, however, induced IgM titers against GD3-L of 1/40–1/1,280 in all patients and IgG titers of 1/160–1/1,280 in 4 patients (Table II). These antibodies also strongly cross-reacted with GD3. Peak reciprocal IgM titers of 40–1,280 were seen against GD3 in 5 of 6 patients and peak reciprocal IgG titers

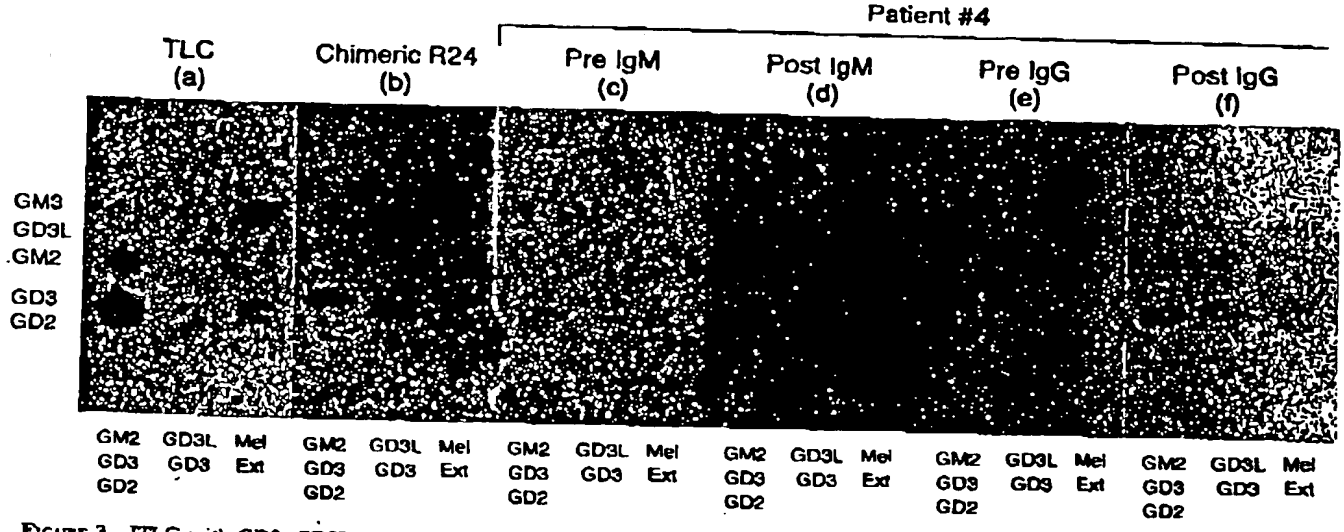


FIGURE 3 – ITLC with GD3, GD3L, GM2, GD2 and melanoma extract and sera from patient vaccinated with GD3-L-KLH. Chimeric MAb R24 was used as a control.

TABLE III – ANTIBODY RESPONSE OF PATIENTS AFTER VACCINATION WITH GD3-L-KLH AS DETERMINED BY DOT-BLOT[1]

Patient number		IgM							IgG						
		GD3-L	GD3	GD2	GM1	GM2	GM3	FGM1	GD3-L	GD3	GD2	GM1	GM2	GM3	FGM1
1	Pre	−	−	−	+	−	−	±	−	−	−	−	−	−	−
	Post	+++	+++	−	+	−	+	+	+++	+++	−	−	−	−	−
2	Pre	−	−	−	+	+	−	++	−	−	−	−	−	−	−
	Post	+++	+++	−	+++	+	+++	−	±	−	−	−	−	±	−
3	Pre	±	−	−	±	−	−	+	−	−	−	−	−	−	−
	Post	+++	+++	−	−	++	−	+	+	+	−	±	++	−	−
4	Pre	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	Post	+++	+++	−	−	−	+	−	+++	++	−	−	−	−	−
5	Pre	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	Post	+++	+++	−	−	−	±	−	+++	++	−	−	−	−	−
6	Pre	−	−	−	−	−	±	−	−	−	−	−	−	−	−
	Post	+++	+++	−	±	−	+++	−	+++	++	−	−	−	−	−
R24	Pre	−	−	−	−	−	−	++	−	−	−	−	−	−	−
	Post	+++	+++	+++	−	−	−	++	+++	+++	−	−	−	−	−
696		±	−	−	−	+++	−	−	+++	+++	−	−	−	−	−

[1]Dot-blot immune stains were graded: − (negative), + (weakly positive), ++ (positive) and +++ (strongly positive). FGM1, fucosyl GM1; R24 and 696 are MAbs against GD3 and GM2, respectively.

against GD3 were 160–1,280 in 4 of 6 patients. The ELISA time course of IgM and IgG antibody induction against GD3 with all sera in all 6 patients receiving the GD3-L-KLH vaccines is shown in Figure 2. IgM antibody titers remained higher than IgG titers at most time points, including after the 2 booster vaccinations. In general, titers were no higher after the booster vaccinations then after initial vaccinations.

Immune response with other gangliosides by ITLC and dot-blot

Pre- and post-vaccination sera of all 6 patients receiving the GD3-L-KLH vaccine were tested by ITLC for reactivity with GD3-L, GD3, GD2 and GM2 as well as with melanoma extract. The immune reactivities of pre- and post-vaccination sera from patient 4 along with chimeric MAb R24 are shown in Figure 3. Sera from 5 of 6 patients showed IgM antibody reactivity as strong as patient 4 against GD3-L and GD3. Sera from patients 1, 4, 5 and 6 also showed comparable IgG reactivity with GD3-L and GD3. Most of the IgM antibodies cross-reacted weakly with GM2 and GD2, whereas IgG antibodies showed only weak cross-reactivity with GD2. Both IgM and IgG reactivities were also seen with higher migrating bands in the melanoma extract, GD3-L.

The specificity of ganglioside antibodies detected in patient sera before and after immunization was also determined by dot-blot immune staining on nitrocellulose membranes containing gangliosides GD3-L, GD3, GD2, GM1, GM2, GM3 and Fuc-GM1. The results are summarized in Table III. A strong positive reactivity of 3+ for IgM antibodies against GD3-L and GD3 was seen in the sera of all 6 patients. Reactivity of 3+ for IgG antibodies was seen in 4 patients with GD3-L and GD3. Pre- immunization IgM and IgG antibodies from all patients showed no reactivity with GD3, although some patients had a low pre-immunization IgM antibody level against GM2 and GM1. Reactivity with these gangliosides

TABLE IV - CELL SURFACE REACTIVITY AGAINST SK-MEL-28 CELLS OF PATIENT SERA AFTER VACCINATION WITH GD3-L-KLH[1]

Patient	SK-MEL-28							
	FACS				IA		CDC	
	IgM		IgG					
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	9.5	9.7	11.0	35.5	Neg	Neg	4.3	7.0
2	8.9	9.7	10.7	19.8	Neg	Neg	1.5	3.0
3	5.5	13.6	9.9	23.4	Neg	1:5	1.9	2.3
4	10.5	8.7	9.8	95.8	Neg	1:10	1.0	56.0
5	5.0	4.8	2.3	10.5	Neg	Neg	3.4	4.5
6	9.1	80.2	11.5	36.2	Neg	1:40	5.4	51.9

[1]MAb R24 (IgG_3) showed 96.75% positive cells by FACS and 26.6% lysis on SK-Mel-21 cells.

was increased after vaccination in 1 pstient each against GM1, Fuc-GM1, GM2 and GD2 and in 2 patients against GM3.

Reactivity of antisera with tumor cells

The cell surface reactivity of peak titer post-immunization antibodies was tested on GD3 positive SK-MEL-28 melanoma cells by FACS, IA and CDC assays. The results with sera from patients receiving the GD3-L-KLH vaccine are summarized in Table IV. The percent positive cells by FACS with pre-vaccination sera were low. Sera from patient 6 showed strongly increased IgM reactivity against SK-MEL-28 cells by FACS, and sera from 4 of 6 patients showed IgG reactivity of over 20% after vaccination. Sera from 3 patients showed post-immunization IA reactivity against SK-MEL-28, and sera from 2 patients showed more than 50% CDC against SK-MEL-28. Under similar conditions, specific release with MAb R24 was 26.6%. Post-vaccination sera in the absence of complement and complement without sera were not cytotoxic.

Antibody specificity determined by inhibition

Two different types of inhibition assays were carried out to define further the specificity of GD3 antibodies in immune sera: (1) incubation of sera with GD3, GD2, GM2 or GM1 and testing back against GD3 by ELISA; and (2) incubation of sera with GD3 and testing back against SK-MEL-28 by FACS. A sample experiment demonstrating the inhibition of IgM and IgG ELISA reactivity for patient 6 (who had shown IgM reactivity against GD2 by dot-blot and ITLC) is shown in Figure 4. The results obtained from all 6 patients are summarized in Table V. The results indicate that GD3 inhibited anti-GD3 IgM at least 10-fold more efficiently than GD2, whereas GM2 and GM1 exhibited no inhibition. The anti-GD3 IgG antibodies were specific for GD3 alone; no other ganglioside inhibited GD3 reactivity.

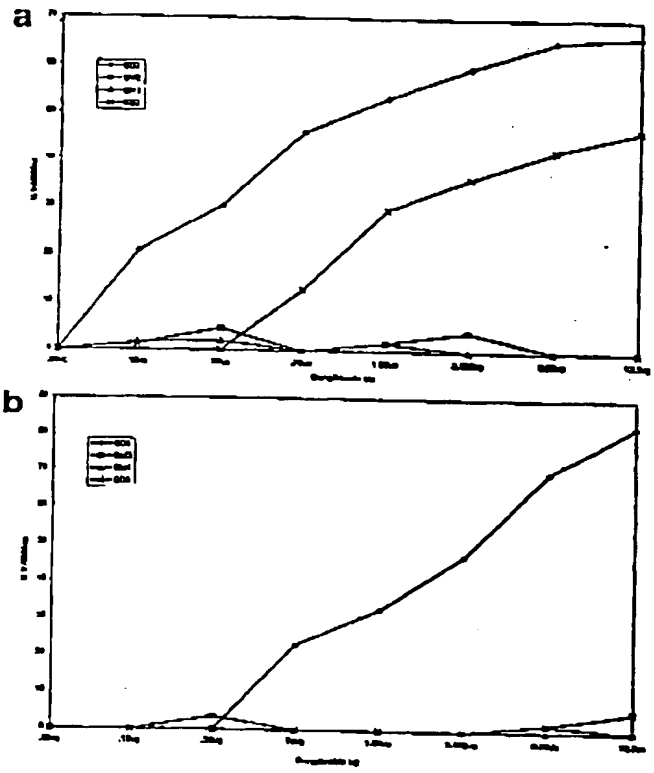


FIGURE 4 – Inhibition of ELISA reactivity of anti-GD3 IgM *(a)* and IgG *(b)* antibody with GD3, GD2, GM1 and GM2 gangliosides (patient 6).

DISCUSSION

Of the melanoma gangliosides considered to be potential targets for immunotherapy, GD3 is the most abundant but also the least immunogenic. Its potential as a target for passive immunotherapy has been documented in patients treated with R24, a murine MAb recognizing GD3. Regression of melanoma metastases after R24 treatment has been demonstrated at several different centers (reviewed by Jurcic *et al.*, 1997; Scheinberg and Chapman, 1995). We have spent considerable effort over the years trying to construct an effective vaccine against GD3 (Livingston, 1995; Ritter *et al.*, 1990*b*, 1991). Our initial approaches were to vaccinate melanoma patients with melanoma cells, whole cell lysates or with purified ganglioside adsorbed to BCG. Using these methods, we were able to induce antibody against GM2 but not against GD3 (Helling *et al.*, 1995; Livingston *et al.*, 1982: Ritter *et al.*, 1991). These experiences led us to search for ways to improve the poor immunogenicity of GD3. Several reports indicated that chemical modification of gangliosides could augment their immunogenicity. We prepared a series of synthetic ganglioside congeners and adhered them to BCG to induce antibody in laboratory animals and melanoma patients (Ritter *et al.*, 1990*a*,*b*, 1991). GD3 amide, GD3 gangliosidol, GD3-L I and II and GD3 acetylated at various sites were all more immunogenic than GD3, but the increased antibody titers induced by these synthetic congeners of GD3 were not reactive with unmodified GD3 or melanoma cells (Ritter *et al.*, 1990*a*,*b*). Low-titer GD3 reactive autoantibodies have been reported in the serum of some melanoma patients after vaccination with irradiated melanoma cells (Ravindranath *et al.*, 1989), but less frequently and far lower titer than antibodies against GM2 and GD2, and no cell surface reactivity could be demonstrated. However, human MAbs reactive with GD3 have been generated (Yamaguchi *et al.*, 1987). It was clear that GD3 was a poor immunogen in humans, but never the less could be recognized by the human immune system.

Immunization of mice with GM3-L induced antibodies that cross-reacted with unmodified GM3 (Nores *et al.*, 1987) and a

TABLE V – INHIBITION OF GD3 ANTIBODY ACTIVITY WITH DIFFERENT GANGLIOSIDES[1]

Sera	GD3 target					
	IgM			IgG		
	GD3	GD2	GM2	GD3	GD2	GM2
Patient 1*	3+	2+	2+	NT[2]	NT	NT
Patient 2*	1+	1+	0	NT	NT	NT
Patient 3	1+	0	0	NT	NT	NT
Patient 4	4+	1+	0	4+	0	0
Patient 5*	3+	0	0	NT	NT	NT
Patient 6	4+	2+	0	4+	0	0

[1]Grading scale: percent inhibition of ≥85% was given a grade of 4+; 70%–85% 3+; 40%–70% 2+; 20%–40% 1+; below 20% 0. GM1 did not inhibit any sera.–[2]NT: not tested.–*Inhibiting on sera with ≥ 1/40.

murine MAb reactive with both GM3 and GM3-L has been generated (Dohi *et al.*, 1988). Murine MAbs reactive with other ganglioside lactones are also reactive with the parent ganglioside (Bosslet *et al.*, 1989; Dohi *et al.*, 1988; Tai *et al.*, 1988). Serum antibodies induced by immunization of mice with GD3-L1 (the lactone ring formed between the carboxyl group of sialic acid and the hydroxyl group of the ganglioside) were shown to react with purified GD3 and GD3 expressing human melanoma cells (Ritter *et al.*, 1990*a*). With GD3-L/BCG, we were able to induce low-titer antibodies against GD3-L in 4 of 9 patients. However, these antibodies were exclusively IgM, the response was of short duration and no reactivity against GD3 was seen. These results suggested that GD3-L/BCG was a more potent method of immunizing against GD3 than GD2/BCG, but was not potent enough (Ritter *et al.*, 1991).

Several other approaches have been reported to augment the immunogenicity of carbohydrate antigens (Helling *et al.*, 1995; Ragupathi *et al.*, 1998). Covalent attachment of carbohydrate antigens to immunogenic protein carriers as first suggested for haptens and then carbohydrates is the concept that has been pursued most vigorously, especially in vaccines against infectious diseases. Regarding conjugate vaccines against gangliosides, in our initial studies with GD3 vaccines in the mouse, we established the optimal conjugation method, the optimal carrier protein and the necessity for a potent adjuvant (Helling *et al.*, 1994). The optimal conjugation procedure for GD3 was cleavage of the ceramide double bond with ozone, generation of an aldehyde group and coupling to free ε-amino groups of the lysine of protein by reductive amination. We found that KLH was the optimal carrier and QS-21, a homogeneous saponin fraction purified from the bark of *Quillaja saponaria*, the most effective adjuvant (Helling *et al.*, 1994). Mice vaccinated with GD3-KLH conjugate plus QS-21 had higher titer IgM antibodies and consistent production of high IgG antibody titers. The superior immunogenicity of the KLH conjugate vaccine plus QS-21 has also been demonstrated in melanoma patients with GM2-KLH (Helling *et al.*, 1995).

Putting together the increased immunogenicity of KLH conjugate plus QS-21 vaccines, the basic ability of the human immune system to produce antibodies recognizing GD3 and the increased immunogenicity of ganglioside GM3-L compared to GM3 in terms of anti-GM3 antibodies, it seemed reasonable to reevaluate the immunogenicity of GD3 and GD3-L using KLH conjugate plus QS-21 vaccines. Others have described clinical trials that occasionally induced antibodies against GD3 in patients (Portoukalian *et al.*, 1991; Ravindranath *et al.*, 1988). We report here an immunization procedure that resulted in the production of IgM and IgG antibodies against GD3 and tumor cells expressing GD3 in the majority of immunized patients. The antibodies produced in response to immunization with GD3-L were specific for GD3-L but also cross-reacted significantly with purified melanoma GD3, GD3 isolated from bovine buttermilk and GD3 on the melanoma cell surface. This is in contrast to the experience of others with MAbs raised against GD3-L which reacted with GD3-L but not with GD3 (Kawashima *et al.*, 1993, 1994). GD3-KLH failed to induce antibody against GD3, suggesting that tolerance to GD3 as a consequence of expression of GD3 on a variety of normal human tissues could be broken by GD3-L but not by GD3. This may be because GD3-L is expressed at lower levels on normal tissues and so is more easily recognized by the immune system, or because GD3-L is a more rigid, less flexible molecule that consequently is a stronger immunogen.

REFERENCES

Bosslet, K., Mennel, H.D., Rodden, F., Bauer, B.L., Wagner, F., Altmannsberger, A., Sedlacek, H.H. and Wiegandt, H., Monoclonal antibodies against epitopes on ganglioside GD_3 and its lactones. Markers for gliomas and neuroblastomas. *Cancer Immunol. Immunother.*, 29, 171–178 (1989).

Dohi, T., Nores, G. and Hakomori, S., An IgG_3 monoclonal antibody established after immunization with GM3 lactone: immunochemical specificity and inhibition of melanoma cell growth *in vitro* and *in vivo*. *Cancer Res.*, 48, 5680–5685 (1988).

Hamilton, W.B., Helling, F., Lloyd, K.O. and Livingston, P.O., Ganglioside expression on human malignant melanoma assessed by quantitative immune thin-layer chromatography. *Int. J. Cancer*, 53, 566–573 (1993).

Helling, F., Shang, A., Calves, M., Zhang, S., Ren, S., Yu, R.K., Oettgen, H.F. and Livingston, P.O., GD3 vaccines for melanoma: superior immunogenicity of keyhole limpet hemocyanin conjugate vaccines. *Cancer Res.*, 54, 197–203 (1994).

Helling, F., Zhang, S., Shang, A., Adluri, S., Calves, M., Koganty, R., Longenecker, B.M., Yao, T.J., Oettgen, H.F. and Livingston, P.O., GM2-KLH conjugate vaccine: increased immunogenicity in melanoma patients after administration with immunological adjuvant QS-21. *Cancer Res.*, 55, 2783–2788 (1995).

Jurcic, J.G., Scheinberg, D.A. and Houghton, A.N., Monoclonal antibody therapy of cancer. *In* H.M. Pinedo, D.L. Longo and B.A. Chabner (eds.), *Cancer chemotherapy and biological response modifiers annual 17*, pp. 195–216, Elsevier, Amsterdam (1997).

Kawashima, I., Kotani, M., Ozawa, H., Suzuki, M. and Tai, T., Generation of monoclonal antibodies specific for ganglioside lactones: evidence of the expression of lactone on human melanoma cells. *Int. J. Cancer*, 58, 263–268 (1994).

Kawashima, I., Ozawa, H., Kotani, M., Suzuki, M., Kawano, T., Gombuchi, M. and Tai, T., Characterization of ganglioside expression in human melanoma cells: immunological and biochemical analysis. *J. Biochem.*, 114, 186–193 (1993).

Kensil, C.R., Patel, U., Lennick, M. and Marciani, D., Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria*, *Molina* cortex. *J. Immunol.*, 146, 431–437 (1991).

Livingston, P.O., Approaches to augmenting the immunogenicity of melanoma gangliosides: from whole melanoma cells to ganglioside-KLH conjugate vaccines. *Immunol. Rev.*, 145, 147–166 (1995).

Livingston, P.O., Adluri, S., Helling, F., Yao, T.-J., Kensil, C.R., Newman, M.J. and Marciani, D., Phase 1 trial of immunological adjuvant QS-21 with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma. *Vaccine*, 12, 1275–1280 (1994).

LIVINGSTON, P.O., WATANABE, T., SHIKU, H., HOUGHTON, A.N., ALBINO, A., TAKAHASHI, T., RESNICK, L.A., MICHITSCH, R., PINSKY, C.M., OETTGEN, H.F. and OLD, L.J., Serological response of melanoma patients receiving melanoma cell vaccines. I. Autologous cultured melanoma cells. *Int. J. Cancer*. 30, 413–422 (1982).

NORRIS, G.A., DOHI, T., TANIGUCHI, M. and HAKOMORI, S., Density-dependent recognition of cell surface GM3 by a certain anti-melanoma antibody, and GM3 lactone as a possible immunogen: requirements for tumor-associated antigen and immunogen. *J. Immunol.*, 139, 3171–3176 (1987).

PORTOUKALIAN, J., CARREL, S., DORÉ, J.-F. and RÜMKE, P., Humoral immune response in disease-free advanced melanoma patients after vaccination with melanoma-associated gangliosides. *Int. J. Cancer*, 49, 893–899 (1991).

PUKEL, C.S., LLOYD, K.O., TRAVASSOS, L.R., DIPPOLD, W.G., OETTGEN, H.F. and OLD, L.J., GD3, A PROMINENT GANGLIOSIDE OF HUMAN MELANOMA. Detection and characterization by a mouse monoclonal antibody. *J. exp. Med.*, 155, 1133–1147 (1982).

RAGUPATHI, G., Carbohydrate antigens as targets for active specific immunotherapy. *Cancer Immunol. Immunother.*, 43, 152–157 (1996).

RAGUPATHI, G., KOGANTY, R.R., QIU, D.X., LLOYD, K.O. and LIVINGSTON, P.O., A novel and efficient method for synthetic carbohydrate conjugate vaccine preparation: synthesis of sialyl Tn KLH conjugate using a 4-(4-*N*-maleimidomethyl) cyclohexane-1-carboxyl hydrazide (MMCCH) linker arm. *Glycoconjugate J.*, 15, 217–221 (1998).

RAVINDRANATH, M.H., MORTON, D.L. and IRIE, R.F., An epitope common to gangliosides O-acetyl-GD3 and GD3 recognized by antibodies in melanoma patients after active specific immunotherapy. *Cancer Res.*, 49, 3891-3897 (1989).

RAVINDRANATH, M.H., PAULSON, J.C. and IRIE, R.F., Human melanoma antigen O-acetylated ganglioside GD3 is recognized by *Cancer antennarius* lectin. *J. biol. Chem.*, 263, 2079–2086 (1988).

RITTER, G., BOOSFELD, E., ADLURI, R., CALVES, M., OETTGEN, H.F., OLD, L.J. and LIVINGSTON, P., Antibody response to immunization with ganglioside GD3 and GD3 congeners (lactones, amide and gangliosidol) in patients with malignant melanoma. *Int. J. Cancer*, 48, 379–385 (1991).

RITTER, G., BOOSFELD, E., CALVES, M.J., OETTGEN, H.F., OLD, L.J. and LIVINGSTON, P.O., Antibody response to immunization with purified GD3 ganglioside and GD3 derivatives (lactones, amide and gangliosidol) in the mouse. *Immunobiology*, 182, 32–43 (1990*a*).

RITTER, G., BOOSFELD, E., MARKSTEIN, E., YU, R.K., REN, S.L., STALLCUP, W.B., OETTGEN, H.F., OLD, L.J. and LIVINGSTON, P.O., Biochemical and serological characteristics of natural 9-O-acetyl GD3 from human melanoma and bovine buttermilk and chemically O-acetylated GD3. *Cancer Res.*, 50, 1403–1410 (1990*b*).

SCHEINBERG, D.A. and CHAPMAN, P.B., Therapeutic applications of monoclonal antibodies for human disease. *In:* J.R. Birch and E.S. Lennox (eds.), *Monoclonal antibodies. Principles and applications*, pp. 45–105. John Wiley & Sons, New York (1995).

SHIKU, H., TAKAHASHI, T., OETTGEN, H.F. and OLD, L.J., Cell surface antigens of human malignant melanoma. II. Serological typing with immune adherence assays and definition of two new cell surface antigens. *J. exp. Med.*, 144, 873–881 (1976).

SVENNERHOLM, L., Chromatographic separation of human brain gangliosides. *J. Neurochem.*, 10, 613–623 (1963).

TAI, T., KAWASHIMA, I., TADA, N. and IKEGAMI, S., Different reactivities of monoclonal antibodies to ganglioside lactones. *Biochim. biophys. Acta*, 958, 134–138 (1988).

YAMAGUCHI, H., FURUKAWA, K., FORTUNATO, S.R., LIVINGSTON, P.O., LLOYD, K.O., OETTGEN, H.F. and OLD, L.J., Cell-surface antigens of melanoma recognized by human monoclonal antibodies. *Proc. nat. Acad. Sci. (Wash.)*, 84, 2416–2420 (1987).

ZHANG, S., HELLING, F., LLOYD, K.O. and LIVINGSTON, P.O., Increased tumor cell reactivity and complement-dependent cytotoxicity with mixtures of monoclonal antibodies against different gangliosides. *Cancer Immunol. Immunother.*, 40, 88–94 (1995).

Immunobiol., vol. 182, pp. 32–43 (1990)

Memorial Sloan-Kettering Cancer Center, New York, U.S.A.

Antibody Response to Immunization with Purified GD3 Ganglioside and GD3 Derivatives (Lactones, Amide and Gangliosidol) in the Mouse*

GERD RITTER**, ERIKA BOOSFELD, MICHELE J. CALVES, HERBERT F. OETTGEN, LLOYD J. OLD, and PHILIP O. LIVINGSTON

Received July 27, 1990 · Accepted in Revised Form October 22, 1990

Abstract

GD3 is the ganglioside most abundantly expressed on the cell surface of human melanoma, and treatment with a monoclonal antibody recognizing GD3 has induced major responses in a small proportion of patients. However, we have been unable to induce production of GD3 antibodies in melanoma patients by active immunization with GD3-expressing melanoma cells or purified GD3. In this report we describe attempts to increase the immunogenicity of GD3 in the mouse by chemical modification. GD3 lactone I and II, GD3 amide and GD3 gangliosidol were synthesized, and the humoral immune response to these derivatives was compared with the response to unmodified GD3. The GD3 derivatives were more immunogenic than GD3. At a low dose all congeners induced an IgM response, with antibody titers higher than those elicited by low-dose GD3. The gangliosidol and amide derivatives also induced an IgG response. IgM antibodies induced by immunization with GD3 lactone I cross-reacted with purified GD3 and GD3-expressing melanoma cells. Titers of GD3 cross-reactive antibodies were slightly higher than after immunization with GD3 itself at the same low dose. IgM and IgG antibodies induced by the other congeners did not cross-react with GD3.

Introduction

In studies of the humoral immune response to ganglioside vaccines in patients with malignant melanoma, it has been shown that GM2 is consistently immunogenic, that GD2 elicits an antibody response only occa-

* This work was supported by a fellowship from the Deutsche Forschungsgemeinschaft (Ri 483/1-1), by grants from the National Institute of Health (CA-43971, CA-08748, CA-33049, NS-11853) and by the Perkins Fund.

** Present address: Fidia Research Laboratories, Dept. of Immunology, 35031 Abano Terme, Via Ponte della Fabbrica 3/A, Italy.

Abbreviations: The designations GM3, GM2, GM1, GD3, GD2 and GD1b are used in accordance with the abbreviated ganglioside nomenclature proposed by SVENNERHOLM (33). *ELISA* = enzyme-linked immunosorbent assay; *HPTLC* = high-performance thin-layer chromatography; *ITLC* = immune thin-layer chromatography; *IA* = immune adherence; *PA* = protein A.

sionally and that GD3 does not induce production of antibody (1–3). GD3, however, is of interest for vaccine construction because it is the most commonly expressed cell surface ganglioside of human melanomas (4–6) and has been a target for the treatment of melanoma with a monoclonal antibody (7). For this reason, we have attempted to increase the immunogenicity of GD3 by chemical modification, and we have examined the humoral immune response to these GD3 derivatives in the mouse. We report that antibodies raised against GD3 lactone I showed cross-reactivity with GD3 in several assays including reactivity with human melanoma cells expressing GD3, while antibodies induced against GD3 lactone II, GD3 amide and GD3 ganglioside cross-reacted with GD3 in ELISA but not in the other assays.

Material and Methods

Gangliosides

GM3, GM2, GM1, GD1b and GD3 were provided by Fidia Research Laboratories (Abano Terme, Italy). GD2 was prepared from GD1b by treatment with bovine testis β-galactosidase (8). Gangliosides of the human melanoma cell line SK-MEL-19 were prepared without saponification or peracetylation by published procedures (9, 10).

Ganglioside derivatives

GD3 lactones were prepared by treating calf brain GD3 with glacial acetic acid as described (11). Lactones were separated according to charge by DEAE-Sephadex A-25 chromatography, eluting lactone II in chloroform/methanol/water 30:60:8 v/v/v and lactone I in 0.05 M NH_4Ac in methanol (12). GD3 amide was obtained by aminolysis of GD3 lactone II (13), followed by treatment with 0.05 M NaOH in methanol for 1 h at 37 °C. GD3 gangliosidols were obtained by reduction of GD3 lactone II with sodium borohydride (14). All derivatives were further purified by Sephadex LH-20 chromatography using chloroform/methanol 1:2 v/v as eluent. The structures of GD3 derivatives are shown in Figure 1.

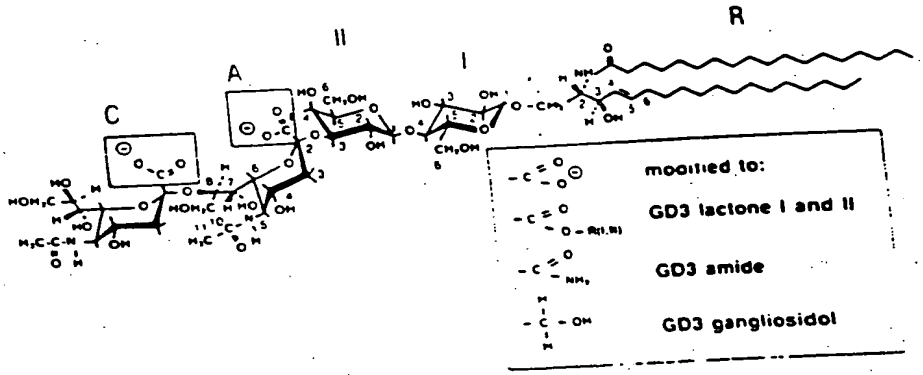


Figure 1. Schematic structures of GD3 derivates used in these studies. For the proposed structures of GD3 lactones refer to reference (11).

Chemicals

HPTLC silica gel plates were obtained from E. Merck (Darmstadt, Germany); nitrocellulose membranes (0.2 μm) from Schleicher and Schuell, Inc. (Keene, NH, USA); Sep-Pak C_{18} cartridges from Waters Associates (Milford, MA, USA); DEAE-Sephadex A-25, Sephadex G-50 and LH-20, 4-chloro-1-napiitiol, and p-nitrophenyl phosphate disodium from Sigma Chemical Co. (St. Louis, MO, USA); cyclophosphamide (Cytoxan) from Mead Johnson (Syracuse, NY, USA).

Monoclonal antibodies and enzymes

Rabbit anti-mouse immunoglobulins conjugated with horseradish peroxidase for ITLC were obtained from Dako Corporation (Santa Barbara, CA, USA), rabbit anti-mouse IgM or IgG conjugated with horseradish peroxidase or alkaline phosphatase from Zymed (San Francisco, CA, USA); MAbs R24, C5 and K9 were generated in our laboratory (15). Bovine testis β-galactosidase was obtained from Dr. George W. Jourdian (Michigan State University, Ann Arbor, MI, USA).

High-performance thin-layer chromatography

TLC analysis was performed on HPTLC silica gel plates. Gangliosides and ganglioside derivatives were separated in chloroform:methanol/0.02% aqueous $CaCl_2$ 60:35:8 (v/v) solvent, and visualized by staining with orcinol:H_2SO_4 or resorcinol/HCl. Two-dimensional TLC was performed as described (13).

Immunization

Six-week old female BALB/c × C57BL/6 F1 mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were injected intraperitoneally with cyclophosphamide (16.15 mg/kg) 3 days before the first immunization. Gangliosides used for immunization were dried in conical tubes and resuspended in distilled water containing the adjuvant *Salmonella minnesota* rough mutant R 595 prepared as described (17). The mixture was lyophylized and emulsified in PBS prior to administration. Mice were injected subcutaneously with a given ganglioside twice, three weeks apart, at a dose of 10 μg glycolipid and 0.5 mg *S. minnesota* R 595 in 100 μl PBS. Mice were bled from the retro-orbital sinus before and two weeks after the first and second vaccine injection. Serum samples for serological testing were stored at −20 °C.

Dot blot immune stains and enzyme-linked immunosorbent assays

These assays were performed as described previously (10).

Table 1. Immunoreactivity of GD3 derivatives[a]

Derivative	anti-GD3 mabs					
	K9		C5		R24	
	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C
GD3	++	+++	++	+++	++	+++
GD3 Amide	−	−	−	−	−	−
GD3 Gangliosidol	−	−	−	−	−	−
GD3 Lactone I	−	++	−	++	−[b]	++
GD3 Lactone II	−	+	−	+	−	+

[a]Determined by ITLC; antibody dilution: 20 μg/ml; anti-GD3 antibodies were incubated overnight. Reactivity was grated as follows: +++ strong, ++ moderate, + weak, − not reactive; [b] minute reactivity.

Immune thin-layer chromatography

Immunostaining of gangliosides and ganglioside derivatives with monoclonal antibodies or mouse sera after separation on HPTLC silica gel glass plates was performed as described (18) with minor modifications (19).

Immune adherence and protein A hemadsorption assays

These assays measure antibody mediated rosetting of human RBC (blood group 0) on target cells. Assays were performed as described (22, 21).

Results

Preparation and characteristics of GD3 lactones, amide and ganglioside

Two major products were obtained by treatment of GD3 with glacial acetic acid. Their TLC-patterns (Fig. 2) corresponded with those of GD3-lactone I and GD3-lactone II as described (11). After mild base treatment, both derivatives co-migrated with the parent GD3. After separation on DEAE-Sephadex A-25 according to charge, lactone I was eluted in the monosialo fraction, whereas lactone II was found in the neutral fraction suggesting that one carboxyl group in lactone I and both carboxyl groups in lactone II were involved in the formation of lactone rings. While no other bands were detected by TLC analysis in the lactone II preparation, the lactone I preparation contained 5–10% GD3. Attempts to remove GD3

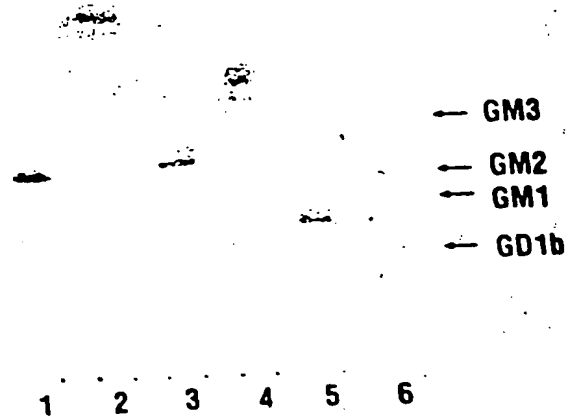


Figure 2. TLC analysis of GD3 and GD3 derivatives used in these studies. GD3 lactone I (Lane 1); GD3 lactone II (Lane 2); GD3 amide (Lane 3); GD3 ganglioside (Lane 4); GD3 (Lane 5); reference gangliosides GM3, GM2, GM1 and GD1b (Lane 6). HPTLC on silica gel plate; running solvent chloroform/methanol/0.02% aqueous $CaCl_2$ 60:35:8 v/v; staining reagent: orcinol/H_2SO_4.

completely from this preparation failed, because some GD3 was always formed again during the purification steps as a consequence of the labile nature of the GD3 lactone I structure. After aminolysis of GD3 lactone II two major products migrating as double bands were detected by TLC, one migrating slightly faster than GM3, the other migrating between GM3 and GM2. The faster migrating product was converted to the slower migrating product by mild base treatment, and only the latter was used in our studies. In contrast to lactones, the GD3 amide showed uniform motility in two-dimensional TLC analysis, after being kept in a chamber saturated with ammonia between the two runs. The GD3 amide preparation was free of GD3 and other products as determined by TLC. The product obtained after reduction of GD3 lactone II appeared as a double band in TLC, migrating slightly faster than GM3. This gangliosidol preparation contained traces of a product migrating with GD3 lactone II and a double band migrating with GM2 (7%). When stained with resorcinol reagent only the major double band developed the typical orange-yellowish color that has been described for gangliosidols (22).

Reactivity of GD3 lactones, amide and gangliosidol with anti-GD3 mAbs

Immune reactivity of these GD3 derivatives with murine mAbs R24, C5 and K9 (recognizing GD3) was determined by ITLC. Results are shown in Table 1. None of the GD3 derivatives reacted with anti-GD3 antibodies when incubated at 4 °C overnight. However, both lactones showed some

Table 2. Antibody response of mice after vaccination with GD3 and GD3 derivatives as determined by ELISA*

Vaccine	Dose	No. of mice	Target	Titers	
				IgM	IgG
GD3	0.1 μg	5	GD3	–	–
	1 μg	5	GD3	–	–
	10 μg	20	GD3	40 (3)	–
	30 μg	5	GD3	1280 (1), 320 (1), 80 (1), 40 (1)	–
GD3-L I	10 μg	20	GD3-L I	1280 (2), 640 (1), 320 (1), 160 (2), 80 (2), 40 (9)	–
			GD3	640 (1), 160 (2), 80 (7), 40 (7)	–
GD3-L II	10 μg	15	GD3-L II	640 (1), 160 (1), 80 (3)	–
			GD3	40 (5)	–
GD3-A	10 μg	15	GD3-A	1280 (4), 640 (2), 320 (3), 160 (2), 80 (4)	> 1280 (12), 160 (2), 80 (1) 40 (1)
			GD3	640 (2), 160 (2), 80 (3), 40 (2)	
GD3-OL	10 μg	15	GD3-OL	1280 (6), 640 (3), 320 (2), 160 (1), 80 (1), 40 (1)	> 1280 (7), 320 (1), 160 (2), 80 (2)
			GD3	80 (1), 40 (1)	–

* Reactivity is expressed in reciprocal titers.

d, because some GD3 was always
teps as a consequence of the labile
After aminolysis of GD3 lactone II
: bands were detected by TLC, one
other migrating between GM3 and
converted to the slower migrating
y the latter was used in our studies.
showed uniform motility in two-
kept in a chamber saturated with
D3 amide preparation was free of
l by TLC. The product obtained
eared as a double band in TLC,
gangliosidol preparation contained
)3 lactone II and a double band
d with resorcinol reagent only the
l orange-yellowish color that has

angliosidol with anti-GD3 mAbs

tives with murine mAbs R24, C5
d by ITLC. Results are shown in
:acted with anti-GD3 antibodies
ver, both lactones showed some

ion with GD3 and GD3 derivatives as

	Titers	
		IgG
		–
		–
		–
0), 80 (1), 40 (1)		–
0), 320 (1),		–
0 (9)		
0 (7), 40 (7)		–
(3)		–
		–
(1),		
		> 1280 (12); 160 (2), 80 (1) 40 (1)
0 (2)		
		> 1280 (7), 320 (1), 160 (2), 80 (2)
		–

Table 3 Antibody response of mice after vaccination with GD3 and GD3 derivatives as determined by dot blot immune stain

Target	Vaccine[1]									
	GD3		GD3-L I		GD3-L II		GD3-A		GD3-OL	
	Number of reactive mice[2]									
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
GM3	1	–	2	–	–	–	–	–	–	–
GM2	–	–	–	–	–	–	–	–	–	–
GM1	–	–	–	–	–	–	–	–	–	–
GD3	4	–	11	–	3	–	1	–	2	–
GD2	–	–	–	–	–	–	–	–	–	–
GD1b	–	–	–	–	–	–	–	–	1	–
GD3-L I	–	–	11	–	–	–	–	–	1	–
GD3-L II	–	–	–	–	1	–	–	–	–	–
GD3-A	–	–	–	–	–	–	15	15	12	–
GD3-OL	2	–	1	–	–	–	4	–	12	7

[1] Each vaccine contained 10 μg ganglioside.
[2] GD3 and GD3-L I were tested in groups of 20 mice. GD3-L II, GD3-A and GD3-OL in groups of 15 mice.

reactivity when incubated with GD3 antibodies at room temperature overnight. It can not be excluded, however, that this reactivity was due to degradation of GD3 lactone to GD3, which we have shown to occur after overnight incubation at room temperature, but not at 4 °C (23).

Immunogenicity of GD3 and GD3 derivatives in the mouse.

The antibody response of mice after immunization with GD3 or GD3 derivatives was analyzed by ELISA (Table 2), dot blot immune stain (Table 3) and by ITLC (Fig. 3).

GD3: Three of 20 mice immunized with 10 μg GD3 responded with production of low titer IgM antibodies against GD3 as determined by ELISA and dot blot immune stain, but not ITLC. Immunization with 0.1 μg GD3 or 1 μg GD3 did not elicit detectable antibody production, while mice immunized with 30 μg GD3 (a dose previously shown to be immunogenic (24)) responded with production of medium titer IgM antibodies against GD3 as determined by ELISA and dot blot immune stains. No IgG antibodies were detected.

GD3 lactone I: Seventeen of 20 mice immunized with 10 μg GD3 lactone I produced IgM antibodies to GD3 lactone I as detected by ELISA, and eleven of the 20 sera were also reactive by dot blot immune stain. Reactivity of these sera with calf brain GD3 was as follows: ELISA 17/20, dot blot immune stain 11/20. Reactivity with human melanoma-derived GD3 was also detected by ITLC. The sera showed no reactivity with GD3 lactone II or other gangliosides. No IgG antibodies were detected.

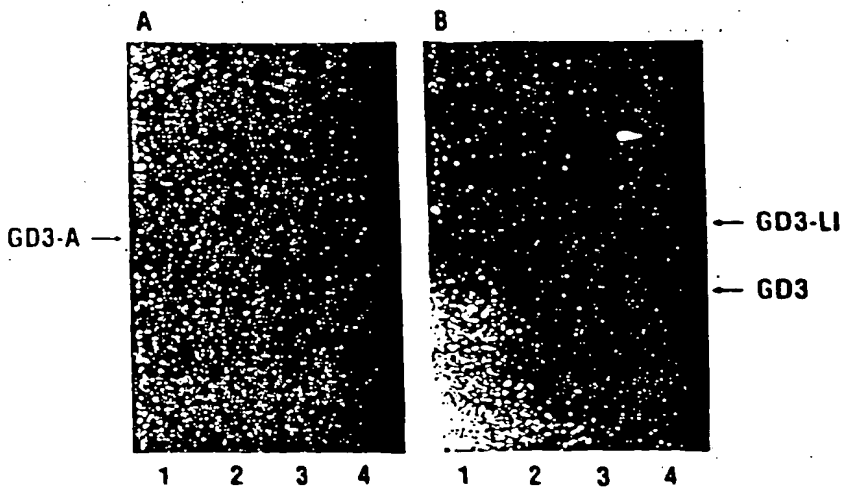
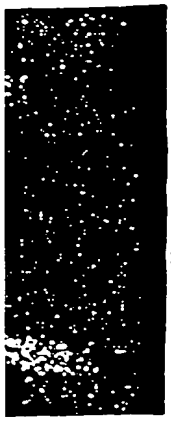


Figure 3. ITLC of mouse antisera induced after immunization with GD3 amide (A) and GD3 lactone I (B). Gangliosides extracted from human melanoma cell line SK-MEL 19 (Lane 1); immunogen: GD3 amide (A) or GD3 lactone I (B) (Lane 2); GD3 from calf brain (Lane 3); reference gangliosides GM3, GM2, GM1 and GD1b (Lane 4). HPTLC on silica gel plates; running solvent chloroform/methanol/0.02% aqueous $CaCl_2$ 60:35:8 v/v; serum dilution 1:75; staining peroxidase and 4-chloro-1-naphthol.

GD3 lactone II: After immunization with 10 μg GD3 lactone II, five of 15 mice developed low titer IgM antibodies against the immunogen and GD3 by ELISA. None of these sera showed reactivity with GD3 lactone II or GD3 by dot blot immune stain or ITLC. These sera showed no reactivity with GD3 lactone I or other gangliosides. Again, no IgG responses were detected.

GD3 amide: Of the GD3 congeners tested, GD3 amide elicited the strongest antibody response. All 15 mice immunized with 10 μg GD3 amide responded with production of high-titer IgM and IgG antibodies reactive with the immunogen as determined by ELISA and dot blot immune stain. Nine of the IgM sera also reacted with GD3 by ELISA, but only one was reactive with GD3 when tested by dot blot immune stain. ITLC revealed only reactivity with the immunogen. IgM antibodies, but not IgG antibodies, showed reactivity with GD3 gangliosidol but not with other gangliosides as determined by dot blot immune stain.

GD3 gangliosidol: After immunization with 10 μg GD3 gangliosidol IgM antibodies to GD3 gangliosidol were detected by ELISA in 14/15 mice and by dot blot immune stain in 12/15 mice, IgG antibodies in 12/15 and 7/15 mice, respectively. Two of the IgM sera also reacted with GD3 in dot blot immune stain tests. Some sera also showed reactivity with GD3 amide by dot blot immune stain and ITLC.



3 4

ion with GD3 amide (A) and GD3
na cell line SK-MEL 19 (Lane 1);
2); GD3 from calf brain (Lane 3);
e 4). HPTLC on silica gel plates;
aCl, 60:35:8 v/v; serum dilution

μg GD3 lactone II, five of
ainst the immunogen and
tivity with GD3 lactone II
sera showed no reactivity
no IgG responses were

GD3 amide elicited the
with 10 μg GD3 amide
IgG antibodies reactive
ot blot immune stain.
SA, but only one was
ain. ITLC revealed
but not IgG anti-
not with other

GD3 ganglioside
SA in 14/15 mice
es in 12/15 and
GD3 in dot
GD3 amide

No antibodies were induced in mice immunized with R595 alone as tested by ELISA. The results indicate that these GD3 derivatives are significantly more immunogenic in the mouse than unmodified GD3 or any other ganglioside we have tested (24).

Cell surface reactivity of sera from immunized mice with human melanoma cells

Immune sera were tested for cell surface reactivity with melanoma cells expressing high levels of GD3 (SK-MEL-19), moderate GD3 (SK-MEL-28), or low GD3 (SK-MEL-31). Mice immunized with 0.1 or 1 μg GD3 showed no reactivity, mice immunized with 10 μg GD3 some reactivity, and mice immunized with 30 μg GD3 showed good reactivity (Table 4). Of the GD3 derivatives, only GD3 lactone I induced antibodies reactive with human melanoma cells expressing GD3 on their cell surface. Although some sera from mice immunized with other GD3 derivatives were reactive with GD3 in ELISA, they showed no reactivity with melanoma cells.

Discussion

The ganglioside GD3 is abundantly expressed on the cell surface of melanomas and therefore a potential target for immunological attack. Treatment with a monoclonal antibody-recognizing GD3 has induced regression of melanoma metastases in a small proportion of patients (7). Attempts at inducing an antibody response in melanoma patients by active immunization with GD3 have failed, however (25), and this experience has led us to search for ways in which the immunogenicity of GD3 could be

Table 4. Cell surface reactivity of antisera induced by GD3 and GD3 derivatives in the mouse with three human melanoma cell lines[1]

Vaccine	Dose	Target cell line[2]		
		SK-MEL-19	SK-MEL-28	SK-MEL-31
GD3	0.1 μg	0/5	0/5	0/5
	1 μg	0/5	0/5	0/5
	10 μg	3/20	3/20	0/20
	30 μg	4/5	4/5	1/5
GD3-L I	10 μg	7/20	4/20	1/20
GD3-L II	10 μg	1/15	0/15	1/15
GD3-A	10 μg	0/15	0/15	0/15
GD3-OL	10 μg	0/15	0/15	0/15

[1] Reactivity was determined by IA and PA and is expressed in number of reactive mice immunized with a given vaccine. No reactivity by PA was observed.
[2] Level of GD3 expression on cell surface: SK-MEL-19 high; SK-MEL-28 moderate; SK-MEL-31 low.

increased. Several reports indicate that chemical modification may augment the immunogenicity of ganglioside molecules. Ganglioside derivatives that have been previously reported to induce antibody production more readily than the parent molecules include GM1 methylester, GM1 gangliosidol and GM1-N-methylamide (26, 27), GM3 lactone (14, 28) and O-acetylation products of GD3 (10).

We report here that modifications of GD3 resulting in enhanced immunogenicity include the lactone I, amide and gangliosidol congeneres. The changes in the molecular structure of GD3 involve loss of charge and altered configuration, hydrophobicity, rigidity and stability. Which of these changes are involved in enhancing immunogenicity is not known. One feature of structurally modified gangliosides is their reduced susceptibility to enzymatic action (26, 29, 30). Higher immunogenicity might result from greater resistance to metabolic degradation, making a lower dose of a GD3 derivative equivalent to an higher dose of GD3. Alternatively, increased immunogenicity might be the consequence of presenting molecular conformations not known to be expressed in mammalian tissue. We have found that the differential expression of individual gangliosides in normal tissues of mice and humans is inversely proportional to their ability to elicit antibody production in these two species (24), suggesting that normal tissue expression rather than chemical structure determines ganglioside immunogenicity.

We were initially impressed by the high-titer antibodies against GD3 detected by ELISA after immunization with some GD3 derivatives (Table 2). However, further testing by dot blot immune stain on nitrocellulose or immune thin-layer chromatography on silica gel plates showed no reactivity with GD3. In our hands, results of dot blot immune stains on nitrocellulose and immune thin-layer chromatography on silica gel plates correlated much better with cell surface reactivity than results of ELISA. Others have also reported that ganglioside antisera show reactivity in ELISA on plastic surfaces but not in other types of tests (31). It appears that epitopes expressed by purified gangliosides in ELISA wells are not necessarily the same epitopes expressed on other artificial matrices or on the cell surface.

In our studies, antisera raised with GD3 amide or GD3 gangliosidol were highly specific for the respective immunogen and showed almost no reactivity with GD3, while antibodies induced by GD3 lactone I (but not lactone II) were equally reactive with GD3 lactone I and with unmodified GD3. Furthermore, these antibodies were reactive with human melanoma cells expressing GD3 on their cell surface, but not reactive with melanoma cells not expressing GD3. Only the GD3 lactone I configuration appeared to be close enough to that of GD3 to induce a crossreactive immune response. Similar observations have been made with GM3. Yu et al. have suggested that the increased hydrophobicity and the more rigid structure of GM3 lactone as compared with native GM3 might favor immunological

recognition (32), and Noris et al. (14, 28) have shown that an immune response against GM3 could be induced with GM3 lactone but not with unmodified GM3.

Our findings suggest that chemical modifications of melanoma gangliosides may increase their immunogenicity and that some chemically modified melanoma gangliosides can be used to construct immunogenic vaccines for immunization of patients with melanoma. We need to keep in mind, however, that the human immune system may not recognize the same epitopes that are recognized in the mouse. Studies investigating the immune response of patients with melanoma to immunization with GD3 derivatives are now underway.

References

1. Livingston, P. O., E. J. Natoli, M. JonesCalves, E. Stockert, H. F. Oettgen, and L. J. Old. 1987. Vaccines containing purified GM2 ganglioside elicit GM2 antibodies in melanoma patients. Proc. Natl. Acad. Sci. USA. 84: 2911.
2. Tai, T., L. D. Cahan, T. Tsuchida, R. E. Saxton, R. F. Irie, and D. L. Morton. 1985. Immunogenicity of melanoma-associated gangliosides in cancer patients. Int. J. Cancer 35: 607.
3. Livingston, P. O., E. Kaelin, C. M. Pinsky, H. F. Oettgen, and L. J. Old. 1985. IV. Serological response in stage II melanoma patients receiving allogeneic melanoma cell vaccines. Cancer 56: 2194.
4. Tsuchida, T., R. H. Ravindranath, R. E. Saxton, and R. F. Irie. 1987. Gangliosides of human melanoma: altered expression in vivo and in vitro. Cancer Res. 47: 1278.
5. Welt, S., E. A. Carswell, C.-W. Vogel, H. F. Oettgen, and L. J. Old. 1987. Immune and nonimmune effector functions of IgG3 mouse mAb R24 detecting the disialoganglioside GD3 on the surface of melanoma cells. Clin. Immunol. Immunopath. 45: 214.
6. Carubia, J. M., R. K. Yu, L. J. Macala, J. M. Kirkwood, and J. M. Varga. 1984. Gangliosides of normal and neoplastic melanocytes. Biochem. Biophys. Res. Comm. 120: 500.
7. Houghton, A. N., M. Mintzer, C. Cordon-Cardo, S. Welt, B. Fliegel, S. Vadhan, E. Carswell, M. R. Melamed, H. F. Oettgen, and L. J. Old. 1985. Mouse monoclonal IgG3 antibody detecting GD3 ganglioside: a phase I trial in patients with malignant melanoma. Proc. Natl. Acad. Sci. USA. 82: 1242.
8. Cahan, L. D., R. F. Irie, R. Singh, A. Cassidenti, and J. C. Paulson. 1982. Identification of a human neuroectodermal tumor antigen (OFA-I-2) as ganglioside GD2. Proc. Natl. Acad. Sci. USA. 79: 7629.
9. Momoi, T., and H. Wiegandt. 1980. Separation and micro-detection of oligosaccharides of GSL by HP-cellulose thin-layer chromatography-autoradiography. Hoppe-Seyler's Z. Physiol. Chem. 361: 1209.
10. Ritter, G., E. Boosfeld, E. Markstein, R. K. Yu, S. Ren, W. B. Stallcup, H. F. Oettgen, L. J. Old, and P. O. Livingston. 1990. Biochemical and serological characteristics of natural 9-O-acetyl GD3 from human melanoma, bovine buttermilk and chemically O-acetylated GD3. Cancer Res. 50: 1405.
11. Ando, S., R. K. Yu, J. N. Scarsdale, S. Kusunoki, and J. H. Prestegard. 1989. High resolution proton NMR studies of gangliosides. Structure of two types of GD3 lactones and their reactivity with mAb R24. J. Biol. Chem. 264: 3478.
12. Iwamori, M., and Y. Nagai. 1978. A new approach to the resolution of individual gangliosides by ganglioside mapping. Biochim. Biophys. Acta 528: 257.

13. Sonnino, S., R. Ghidoni, V. Chigorno, M. Masserini, and G. Tettamanti. 1985. Recognition by two-dimensional TLC and densitometric quantification of alkali-labile gangliosides from the brain of different animals. Anal. Biochem. 128: 104.
14. Nores, G. A., T. Dohi, M. Taniguchi, and S. I. Hakomori. 1987. Density-dependent recognition of cell surface GM3 by a certain anti-melanoma antibody, and GM3 lactone as a possible immunogen: J. Immunol. 139: 3171.
15. Dippold, W. G., K. O. Lloyd, L. T. Li, H. Ikeda, H. F. Oettgen, and L. J. Old. 1980. Cell surface antigens of human malignant melanoma: definition of six antigenic systems with monoclonal antibodies. Proc. Natl. Acad. Sci. USA. 77: 6114.
16. Livingston, P. O., A. B. DeLeo, M. Jones, and H. F. Oettgen. 1983. Comparism of approaches for augmenting the serolog. response to the individually spec. methylcholanthrene-induced sarcoma-Meth A. J. Immunol. 131: 2601.
17. Livingston, P. O., M. JonesCalves, and E. J.Natoli. 1987. Approaches to augmenting the immunogenicity of the ganglioside GM2 in mice. Purified GM2 is superior to whole cells. J. Immunol. 138: 1524.
18. Magnani, J. L., D. F. Smith, and V. Ginsburg. 1980. Detection of gangliosides that bind cholera toxin: direct binding of 125I-labeled toxin to thin-layer chromatograms. Anal. Biochem. 109: 399.
19. Ritter, G., W. Krause, R. Geyer, S. Stirm, and H. Wiegandt. 1987. Glycosphingolipid composition of human semen. Arch. Biochem. Biophys. 257: 370.
20. Shiku, H., T. Takahashi, H. F. Oettgen, and L. J. Old. 1976. Cell surface antigens of human malignant melanoma. J. Exp. Med. 144: 873.
21. Pfreundschuh, M., H. Shiku, T. Takahashi, R. Ueda, J. Ransohoff, H. F. Oettgen, and L. J. Old. 1978. Serological analysis of cell surface antigens of malignant human brain tumors. Proc. Natl. Acad. Sci. USA. 75: 5122.
22. MacDonald, D. L., L. M. Patt, and S. I. Hakomori. 1980. Notes on improved procedures for the chemical modification and degradation of glycosphingolipids. J. Lipid Res. 21: 642.
23. Ritter, G., P. O. Livingston, E. Boosfeld, H. Wiegandt, R. K. Yu, H. F. Oettgen, and L. J. Old. 1989. Development of melanoma vaccines: Gangliosides as immunogens. In: Gangliosides and cancer. Oettgen, H. F. (ed.) VCH Verlagsgesellschaft Weinheim West Germany. 301 pp.
24. Livingston, P. O., G. Ritter, and M. J. Calves. 1989. Antibody response after immunization with the gangliosides GM1, GM2, GM3, GD2 and GD3 in the mouse. Cancer Immunol. Immunother. 29: 179.
25. Livingston, P. O., G. Ritter, H. F. Oettgen, and L. J. Old. 1989. Immunization of melanoma patients with purified gangliosides. In: Gangliosides and cancer. Oettgen, H. F. (ed.) VCH Verlagsgesellschaft Weinheim West Germany. 293 pp.
26. Nakamura, K., and S. Handa. 1986. Biochemical properties of N-methyl-amides of sialic acids in gangliosides. J. Biochem. 99: 219.
27. Handa, S., and K. Nakamura. 1984. Modification of sialic acid carboxyl group of gangliosides. J. Biochem. 95: 1323.
28. Dohi, T., G. Nores, and S. I. Hakomori. 1988. An IgG3 mAb established after immunization with GM3 lactone: Immunochemical specificity and inhibition of melanoma cell growth. Cancer Res. 48: 5680.
29. Klein, D., G. Pohlentz, U. Hinrichs, and K. Sandhoff. 1987. Metabolism of ganglioside-amides in cultured human fibroblasts. Biol. Chem. Hoppe' Seyler 368: 1496.
30. Li, S. C., S. Serizawa, Y. T. Li, K. Nakamura, and S. Handa. 1984. Effect of modification of sialic acid on enzymatic hydrolysis of gangliosides GM1 and GM2. J. Biol. Chem. 259: 5409.
31. Gillard, B. K., J. W. Thomas, L. J. Nell, and D. M. Marcus. 1989. Antibodies against ganglioside GT3 in the sera of patients with type I diabetes mellitus. J. Immunol. 142: 3826.
32. Yu, R. K., T. A. W. Koerner, S. Ando, H. C. Yohe, and J. H. Prestegard. 1985. High-

resolution proton NMR studies of gangliosides. III. Elucidation of the structure of GM3 lactone. J. Biochem. 98: 1367.

33. SVENNERHOLM, L. 1963. Chromatographic separation of human brain gangliosides. J. Neurochem. 10: 613.

Dr. GERD RITTER, Fidia Research Laboratories, Department of Immunology, 35031 Abano Terme, Via Ponte della Fabbrica 3/A, Italy